Physical-Chemical Interactions between Bacteria and Fungi

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For my mother

Моей маме

Paranimfen: Agnieszka Muszanska

Evgenia Verovskaya

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Chapter 1

General Introduction

(partially based on: Jarosz L.M.; Ovchinnikova E.S.; Meijler M.M.; Krom B.P. “Microbial spy games and host response: roles of a *Pseudomonas aeruginosa* small molecule in communication with other species”, *PLoS Pathog*en, 2011, 7 : e1002312)

In nature, microorganisms not only form monoculture biofilms but they can also coexist within complex polymicrobial communities, containing both commensals and opportunistic pathogens. As a consequence, biofilm communities in nature are characterized by their own unique combination of strains and species [1, 2]. Polymicrobial communities are mostly found in the oral cavity, intestinal tract, on the skin as well as other sites of the human body and are colonized by diverse bacterial and fungal populations [3, 4]. For survival and reproductive success, interacting organisms in polymicrobial communities often take benefit of each other's presence. For example, β-lactamase–positive *Moraxella catarrhalis* increased tolerance of *Streptococcus pneumonia* to β-lactam antibiotics when they grow together within a biofilm [5]. Lactate produced by *Lactobacilli spp*. in the gastrointestinal tract is utilized by *Megasphera elsdenii* and *Mitsuoka multiaciad* [6]. In contrast, antagonistic interactions between microorganisms in mixed biofilms occur as well, like toxins produced by *Pseudomonas aeruginosa* inhibiting growth of *Staphylococcus aureus*, while *P. aeruginosa* has been suggested to be able to lyse *S. aureus* [7, 8].

Additionally microbial pathogens have developed sophisticated cell–cell communication systems termed quorum sensing (QS). QS is a system that enables microbes to monitor population cell density through the production, secretion, and sensing of small diffusible molecules and it also allow bacteria intercept of critical information about the status of competing microbes and used it for their benefit [9, 10]. In the Gram-negative pathogenic bacterium *P. aeruginosa*, the QS system is perhaps the most complex because several distinct QS sub-systems are hierarchically intertwined at different stages [10, 11]. The best-studied of these systems is the LasI/R, which consists of the LasI protein that catalyzes the synthesis of the diffusible molecule $3OC_{12}HSL$ [10, 12]. Intracellular accumulation of $3OC_{12}HSL$ is sensed by the receptor LasR and induces expression of several

virulence factors, such as exotoxins and proteases, and production of secondary metabolites. Moreover, competing bacterial species have developed ways to detect *P. aeruginosa* through secreted $3OC_{12}HSL$. Specifically, the bacterial pathogen *S. aureus* has been shown to respond to the presence of *P. aeruginosa* [13]. $3OC_{12}HSL$ specifically induces down-regulation of the *sarA* and *agr* genes and consequently, several virulence factors, such as hemolysin, exotoxin, and fibronectin-binding protein, and factors related to biofilm formation are down-regulated in *S. aureus* in response to *P. aeruginosa* presence [10, 13].

Infections related to burn wounds, cystic fibrosis, and periodontal diseases consist commonly of the bacteria *P. aeruginosa* and *S. aureus* and the fungus *Candida albicans*, and represent niches with an active microorganisms interaction [10]. *C. albicans* is a dimorphic fungus and able to switch morphology between the yeast and hyphal forms [14], a property crucial to its pathogenesis. Even though *C. albicans* alone is the fourth leading cause of mortality caused by systemic infections [15, 16], the risk of mortality or severity of disease may increase due to bacterial and fungal cooperation. *C. albicans* ability to interact with its bacterial neighbors has been described extensively. For instance, *P. aeruginosa* adheres to *C. albicans* hyphae but not to the yeast morphology, making only the hyphal morphology susceptible to killing by *P. aeruginosa* [17, 18]. Even though the interaction between these two species is antagonistic, it was found that *C. albicans* colonization of the respiratory tract was associated with an increased risk of *P. aeruginosa* ventilator-associated pneumonia [19]. It was shown that in approximately 20% of bloodstream infections, *C. albicans* was co-isolated with *S. aureus* [20]. *S. aureus* is a Gram-positive coccus that is responsible for a significant and increasing number of hospital infections worldwide due to its ability to develop antibiotic resistance to a number of clinically important antibiotics [21, 22]. Interestingly, co-infection of mice with *C.*

albicans and *S. aureus* demonstrated a synergistic effect [23, 24]. Moreover, Harriott *et al.* [16, 25] demonstrated that in mixed species biofilms with *S. aureus* and *C. albicans* bacteria were protected against the action of antibiotics such as vancomycin.

Despite the fact that interactions between the human fungal pathogen *C. albicans* and different bacteria have been investigated extensively [15, 26], there is still limited information on the interplay between physico- and bio-chemical mechanisms that mediate their interaction. In order to allow interactions between species to occur, individual cells need to come close to each other, a process governed by attractive forces between the cells. Non-specific interactions typically refer to Lifshitz-Van der Waals (LW) forces that are usually attractive and originate from all molecules in the entire cell body. In addition, attractive forces can originate from localized so-called electron-donor/electron-acceptor or Lewis acid/base (AB) forces or electrostatic interactions between stereo-chemical groups, such as in receptor-ligand interactions [27]. Thermodynamic modeling can be used to evaluate the nature of the adhesion forces between two microbial surfaces by calculating the interfacial free energy of the interacting organisms (ΔG_{adh}). The calculated ΔG_{adh} values are based on contact angle measurements with different liquids on the interacting surfaces [28, 29].

In combination with thermodynamic modeling, direct measurement of adhesion forces between microorganisms is possible using atomic force microscopy (AFM). AFM allows us to record an actual adhesion forces that occur between a bacterial probe attached to a flexible tipless cantilever and an microorganism immobilized on a substratum surface (Figure 1). AFM adhesion forces are probed directly between microbial cell surfaces as a function of distance [30, 31].

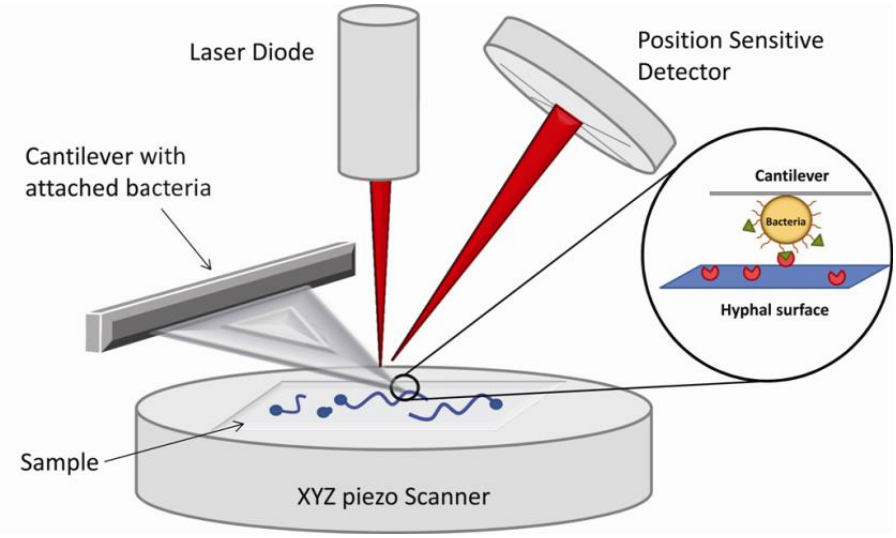

Figure 1 Schematic representation of AFM set-up used. A sample with attached *C. albicans* cells is positioned by a xyz piezo scanner, while a bacterium attached to a tipless AFM cantilever is coming into contact with the hyphal surface on the sample surface. The deflection of the cantilever upon retract is a measure of the adhesion forces between a bacterium and the hyphal surface and is detected by an optical laser. The laser beam is focused on the very end of the cantilever and reflected onto a position sensitive detector from which the adhesion forces can be calculated, provided the mechanical properties of the cantilever are known.

Upon initial approach, a repulsive force is measured, but upon retract attractive forces are measured that decrease with distance until rupture of the bond. Our work combines experiments and modeling approaches for better understanding the initial steps of the interaction between bacteria and fungi in their yeast and hyphae morphologies.

Aim

The aim of this thesis is to reveal physico-chemical and biological mechanisms involved in interactions between the eukaryotic pathogen, *C. albicans* and prokaryotic bacterial pathogens.

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Chapter 2

Force microscopic and thermodynamic analysis of the adhesion between *Pseudomonas aeruginosa* and *Candida albicans*

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ABSTRACT

Pseudomonas aeruginosa expresses a plethora of virulence factors and many species have developed warning systems to detect and evade *P. aeruginosa*. *Candida albicans* detects *P. aeruginosa* by sensing the secreted bacterial quorum sensing molecule $3OC_{12}HSL$ and responds by reverting to the yeast morphology, resistant to killing by *P. aeruginosa*. The aim of this study was to investigate the nature of the adhesion forces between *P. aeruginosa* and different *C. albicans* morphologies, based on surface thermodynamics and atomic force microscopy. Adhesion of *P. aeruginosa* to hyphae was always accompanied by strong adhesion forces, but did not occur to yeast cells. Surface thermodynamics and Poisson analyses of adhesion forces indicated that the outermost mannoprotein-layer on hyphal surfaces created favorable acid-base conditions for adhesion, allowing close approach of *P. aeruginosa*. Removal of these proteins caused unfavorable acid-base conditions, preventing adhesion of *P. aeruginosa*. Yet, favorable acid-base conditions alone are insufficient for mediating adhesion of *P. aeruginosa* to hyphae. *P. aeruginosa* PA14 *lasI*, unable to produce $3OC_{12}HSL$, showed favorable acid-base conditions, but was considerably less adherent to hyphae. However, growth in presence of $3OC_{12}HSL$ restored adhesion. Concluding, the mannoprotein-layer on the hyphal surface ensures favorable interaction conditions through attractive Lifshitz-Van der Waals and acid-base forces, allowing *P. aeruginosa* to closely approach the hyphal surface and interact stereo-chemically with the fungal cell wall for actual adhesion forces to take place. Interestingly, whereas on the one hand $3OC_{12}HSL$ allows the bacterium to interact with hyphae, the same molecule induces changes in acid-base properties of the fungal outermost mannoprotein-layer during transition from the hyphal to yeast morphology, preventing this interaction and allowing *C. albicans* to co-exist with *P. aeruginosa*.

INTRODUCTION

Polymicrobial communities are ubiquitous in nature, including in human health and disease and each community is characterized by its own combination of strains and species [1-3]. The oral cavity is known to host several hundreds of different strains and species [4] that colonize the tooth surface in a spatio-temporally structured manner [5] while the intestinal tract and the skin are also colonized by diverse bacterial and fungal populations [6]. Interacting organisms in polymicrobial communities often take benefit of each other's presence. For metabolic, probiotic or antagonistic interactions between species to occur, cells of both species need to be in close proximity of each other [7]. In order to allow this, individual microorganisms need to adhere to each other, a process governed by attractive forces. Attractive Lifshitz-Van der Waals (LW) forces are operative between any two cell surfaces and originate from all molecules in the entire cell body to yield an overall, non-specific adhesion. In addition, attractive forces can originate from localized acid-base (AB) or electrostatic interactions between stereo-chemical groups, such as in receptor-ligand interactions [8]. Specific interactions can only develop if non-specific forces have brought interacting organisms close enough to each other.

Interactions between the human fungal pathogen *Candida albicans* and bacteria have been investigated extensively [9] but there is limited information on the interplay between physico- and bio-chemical mechanisms that mediate their interaction. Surface thermodynamics [10] can be used to evaluate the nature of the adhesion forces between two microbial surfaces by calculating the surface free energies of the interacting organisms. For example, analysis of the adhesion between *C. albicans* ATCC 10261 and *Streptococcus gordonii* NCTC 7869 indicated that thermodynamically favorable LW forces were ubiquitously present, while AB forces between yeasts and streptococci were only favorable if the yeasts were

grown at 37°C, a growth temperature that stimulates the transition of the yeast to hyphal morphology of the fungus [11].

In addition to a thermodynamic approach, direct measurement of adhesion forces between organisms is possible using atomic force microscopy (AFM). AFM can measure adhesion forces occurring between microbial cell surfaces as a function of distance [12]. A force-distance curve measured with AFM records the adhesion forces between a bacterial probe attached to a flexible cantilever and an organism immobilized on a substratum surface. Upon initial approach, a repulsive force is measured, but upon retract attractive forces are measured that decrease with increasing separation distance until rupture of the bond occurs. AFM has proven to be useful in demonstrating adhesion forces between co-aggregating and non-co-aggregating oral bacterial pairs [13]. Also auto-aggregation of *Enterococcus faecalis* and its inhibition by specific antibodies has been related with adhesion forces between the bacteria as measured using AFM [14]. While surface thermodynamic analyses of LW and AB interactions are often critically viewed, AFM can provide more direct information on the nature of bacterial adhesion forces by means of Poisson analysis of the measured forces [15]. Poisson analysis allows identification of single-bond AB contributions and an overall LW force that together form the adhesion force between two microorganisms [16].

The polymorphic human fungal pathogen *C. albicans* and the Gram-negative human bacterial pathogen *Pseudomonas aeruginosa* are often co-isolated from patients with cystic fibrosis or burn wounds [2, 17]. The interaction between the two species is antagonistic and occurs as an interplay between chemical communication and physical adhesion. Chemical communication is commonly mediated through small diffusible molecules termed quorum sensing (QS) molecules. Chemical communication between *P. aeruginosa* and *C. albicans*

is mediated by the small, secreted molecule 3-oxododecanoyl-L-homoserine lactone ($3OC_{12}HSL$), a QS molecule synthesized by LasI in *P. aeruginosa*. $3OC_{12}HSL$ accumulates in the medium during growth and is sensed by *C. albicans* [18]. *P. aeruginosa* can also physically interact with *C. albicans* as it readily adheres to and forms biofilms on hyphae [18]. Adhesion to hyphae results in rapid lysis and cell death through a currently unidentified mechanism [17]. Interestingly, the yeast morphology is completely resistant to *P. aeruginosa* adhesion and related cell lysis and death [18].

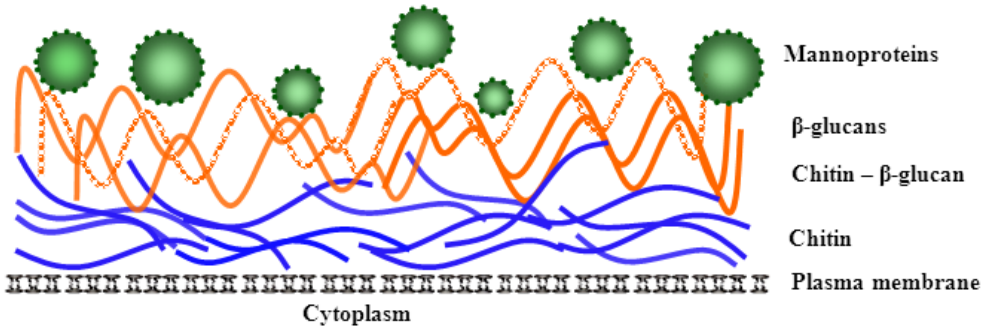

Figure 1 Schematic model of the major cell wall components of *C. albicans*. An inner layer of fibrillar polysaccharides, consisting of chitin (blue), a mixed layer of chitin and β-1,3-glucan (orange) and β-1,6-glucan (dashed orange) is covered with an outermost layer of different mannoproteins (green), covalently attached to the β-glucans (adapted from Chaffin [19]).

The fungal cell wall consists of a layer of polysaccharides, covered with a dense layer of surface proteins, as schematically presented in Figure 1. The mannoprotein layer on top of these polymers contains 38% to 40% of the dry weight of both yeast cells and hyphae, but possesses a different protein composition [19]. Although it is known that the protein composition of this outermost cell surface affects the cell surface hydrophobicity [20] including adhesion of hydrophobic latex microspheres [21] it is unknown how the difference in outer cell surface composition between the yeast and hyphal

morphology affects the surface thermodynamics and adhesion forces with *P. aeruginosa*.

The aim of this study was to investigate the actual physical interaction between *P. aeruginosa* and different morphologies of *C. albicans* based on surface thermodynamics and AFM adhesion forces.

MATERIALS AND METHODS

Strains, growth conditions and harvesting

The fungus *C. albicans* SC5314 [22] and bacterial strains *P. aeruginosa* PA14 [23] (wild type) and the mutant *P. aeruginosa* PA14 *lasI* [24], were used. Strains were grown on tryptone soy broth (TSB, Oxoid, Basingstoke, UK) w/o amino solidified with 1.5% bactoagar. Single colonies from agar plates were used to inoculate 5 ml TSB for bacterial precultures or 5 ml nitrogen base acids (YNB; Difco, Sparks, USA) pH 7, containing 0.5% D-glucose yeast preculture for *C. albicans* precultures [25]. Precultures of *C. albicans* and bacteria were grown at 30°C or 37°C , respectively, for 24 h with rotation (150 rpm) and used to inoculate a main culture (1:50 dilution). Bacterial main cultures were grown for an additional 18 h under the same conditions. *P. aeruginosa* PA14 *lasI* was also grown with 5 µM synthetic $3OC_{12}HSL$, i.e. is at the upper limit of the naturally occurring concentration [24]. Germ-tubes, representing young hyphae, were induced by growing a culture (1:50 dilution) in YNB pH 7.0 for 4 h with rotation (150 rpm) and increasing the growth temperature to 37°C [26]. Germ-tube formation was obtained at 90-95% efficiency under these conditions, as microscopically confirmed. Main cultures were harvested by centrifugation for 5 min at 6,250 x g and 14,800 x g for *P. aeruginosa* and *C. albicans*, respectively, followed by two washes with ultrapure water. In order to analyze the influence of *C. albicans* cell wall mannoprotein layer on actual interaction with bacteria, *C. albicans* hyphal cells were also treated for

30 min at 37 ºC with 2.5 mg/ml of pronase E (Merck) in 10 mM potassium phosphate buffer, pH 7. After treatment, the organisms were rinsed twice with 10 mM potassium phosphate buffer, pH 7.

Adhesion assay

Adhesion of *P. aeruginosa* to *C. albicans* in either the yeast or hyphal morphology was enumerated per unit hyphal length using bright field microscopy (Leica DM4000B). Hyphal formation was induced by growth at 37°C in a 6 or 12 wells plate (Costar, Corning Inc., NY, USA). After 4 h of growth, wells were washed once and 10 mM potassium phosphate buffer, pH 7 was added. Bacteria were added to a final optical density of 0.1 measured at 600 nm. After 90 min of co-incubation at 37°C under static conditions, wells were gently washed twice with phosphate buffer and images were captured at five randomly chosen locations in the wells using a 40x water immersion objective.

Contact angle measurements and surface thermodynamic analysis

To determine microbial cell surface free energies, microorganisms were layered onto 0.45 µm or 3 µm pore-size Millipore filters for bacteria and yeast, respectively using negative pressure. Bacteria were resuspended in demineralized water, while fungi were layered from a suspension in TNMC buffer (1 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$). The filters were left to dry in ambient air, until plateau water contact angles of sessile droplets [27] could be measured, representing a state of cell surface hydration most closely resembling the physiologically occurring one [28]. For each strain, three independently grown cultures were used, while three microbial lawns were prepared out of each culture.

To allow surface thermodynamic analysis [28] contact angles were measured with water, formamide, methylene iodide and 1-bromonaphthalene and the microbial cell surface free energy (γ^{tot}), its LW (γ^{LW}) and AB (γ^{AB}) components and the electron-donating and accepting parameters [29] were calculated. The bacterial and fungal surface free energies were subsequently employed to calculate the free energy of interaction (ΔG_{adh}) between the bacterial (B) and fungal cell-surface (S) in an aqueous suspension (L) and its LW (ΔG_{adh}^{LW}) and AB (ΔG_{adh}^{AB}) components

$$\Delta G_{adh} = \Delta G_{adh}^{LW} + \Delta G_{adh}^{AB} \quad (1)$$

where

$$\Delta G_{adh}^{LW} = \left(\sqrt{\gamma_B^{LW}} - \sqrt{\gamma_S^{LW}}\right)^2 - \left(\sqrt{\gamma_B^{LW}} - \sqrt{\gamma_L^{LW}}\right)^2 - \left(\sqrt{\gamma_S^{LW}} - \sqrt{\gamma_L^{LW}}\right)^2 \quad (2)$$

and

$$\Delta G_{adh}^{AB} = 2\left[\sqrt{\gamma_L^+}\left(\sqrt{\gamma_B^-} + \sqrt{\gamma_S^-} - \sqrt{\gamma_L^-}\right) + \sqrt{\gamma_L^-}\left(\sqrt{\gamma_B^+} + \sqrt{\gamma_S^+} - \sqrt{\gamma_L^+}\right) - \sqrt{\gamma_B^-\gamma_S^+} - \sqrt{\gamma_B^+\gamma_S^-}\right] \quad (3)$$

According to surface thermodynamics, interaction will be favorable if $\Delta G_{adh} < 0$ [10].

Atomic force microscopy

C. albicans was immobilized on glass slides (Menzel, GmbH, Germany), coated with positively charged poly-L-lysine [30]. A fungal suspension was deposited onto

the coated glass and left to settle at room temperature for 20 min. Non-adhering cells were removed by rinsing with demineralized water and the slide was kept hydrated prior to AFM analysis in phosphate buffer. To create a bacterial probe, *P. aeruginosa* was immobilized onto poly-L-lysine treated tipless "V"-shaped cantilevers (DNP-0,Veeco Instruments Inc., Woodbury, NY, USA). To this end, cantilevers were mounted in a micromanipulator under microscopic observation to allow only the tip of the cantilever to be coated. A droplet of poly-L-lysine solution was placed on a glass slide, and the tip of the cantilever was dipped in the droplet for 1 min. After air-drying the cantilever for 2 min, it was dipped in a bacterial suspension for 1 min. Bacterial probes were freshly prepared for each experiment.

Adhesion forces were measured at room temperature in phosphate buffer, using an optical lever microscope (Nanoscope IV, Digital Instruments). For each bacterial probe, force curves were measured after different bond-maturation times up to 60 s on the same, randomly chosen spot on a fungal cell with z-scan rates of less than 1 Hz. To ensure that no bacteria detached from the cantilever during the experiment, control force-distance curves were made with 0 s contact time after each set of measurements. Whenever the "0 s contact time" forces measured deviated more than 0.5 nN from the initial measurement, a bacterial probe was considered damaged and replaced. For each combination of a bacterial strain and fungal–coated glass surface, five different probes were employed on average and the number of bacterial probes used depended on the outcome of the control measurements. Calibration of each cantilever was done using the thermal tuning method (Nanoscope V6.13r1), yielding a range of spring constants from 0.04 to 0.1 (N/ m).

For each bacterial probe, the maximum adhesion forces were plotted as a function of the bond-maturation time Δt, and fitted to

$$F(\Delta t) = F_{0s} + (F_{\infty} - F_{0s})(1 - \exp\{-\Delta t/\tau\}) \quad (4)$$

with F_{0s} being the maximum adhesion force at 0 s contact time, F_{∞} being the maximum adhesion force after bond-maturation, and τ being the characteristic time needed for the adhesion force to strengthen [31]. Since the adhesion forces measured obey a Poisson distribution [16] the adhesion force can be expressed as

$$P(F) = (F_{av})^n \times \frac{\exp(-F_{av})}{n!} \quad (5)$$

with *P(F)* the probability that an adhesion event involving a force *(F)* will occur, F_{av} the average of all adhesion forces, and *n* the number of adhesion forces included. The total adhesion force is comprised of a main peak due to an invariant non-specific force and a variable number of minor peaks n_{AB}, constituted by hydrogen bonds, according to

$$F = n_{AB-bond} f_{AB-bonding} + F_{NS-bonding} \quad (6)$$

where $f_{AB-bonding}$ and $F_{NS-bonding}$ represent the single-bond acid-base contribution and overall non-specific force to the total adhesion force, respectively. Based on Eqs. 5 and 6, the relationship between the force average (μ_F) and variance (σ_F^2) of all adhesion events can be expressed as [16]

$$\mu = n_{AB-bond} \quad f_{AB-bonding} + F_{NS-bonding} \quad (7)$$

$$\sigma_F^2 = \mu_F f_{AB-bonding} - f_{AB-bonding} F_{NS-bonding} \quad (8)$$

According to Eq. 8, a plot of the variance (σ_F^2) versus the force average (μ_F) yields a straight line. Linear regression of σ_F^2 versus μ_F directly decouples the adhesion force into a single-bond AB contribution $f_{AB-bonding}$ and an overall, non-specific force, $F_{NS-bonding}$.

Statistics

Adhesion forces for different fungus-bacterium pairs were compared using non-parametric analyses (Mann-Whitney test). Differences were considered significant when the P-value was < 0.05.

RESULTS

Microscopic enumeration showed that *P. aeruginosa* PA14 adhered to hyphae of *C. albicans* SC5314 (Figure 2A), but not to yeast cells (Figure 2B). For *P. aeruginosa* PA14 *lasI* adhesion to hyphae was almost completely absent (Figure 2C), but *P. aeruginosa* PA14 *lasI*, grown in the presence of the synthetic QS molecule 3OC12HSL (Figure 2D) had a restored expression of surface proteins to the extent that it adhered to hyphae in comparable numbers as the parent strain, *P. aeruginosa* PA14 (Figure 2F). On pronase treated hyphae hardly any *P. aeruginosa* PA14 adhered and the number of adhering bacteria was very similar to the lasI mutant strain (Figure 2E and 2F). Microbial cell surface hydrophobicity, as judged by water contact angles indicated that all cell surfaces were hydrophilic (water contact angles between 25 and 31 degrees), with the exception of *C. albicans* hyphae, which were hydrophobic (Table 1), with a water contact angle of 107 degrees. However, removal of the outer mannoprotein layer by pronase E

treatment reduced the hyphal cell surface hydrophobicity of *C. albicans* SC5314 to a water contact angle of 34 degrees. Accordingly, using contact angles with liquids of different polarities, the LW surface free energy component and the electron-donating and accepting surface free energy parameters were calculated.

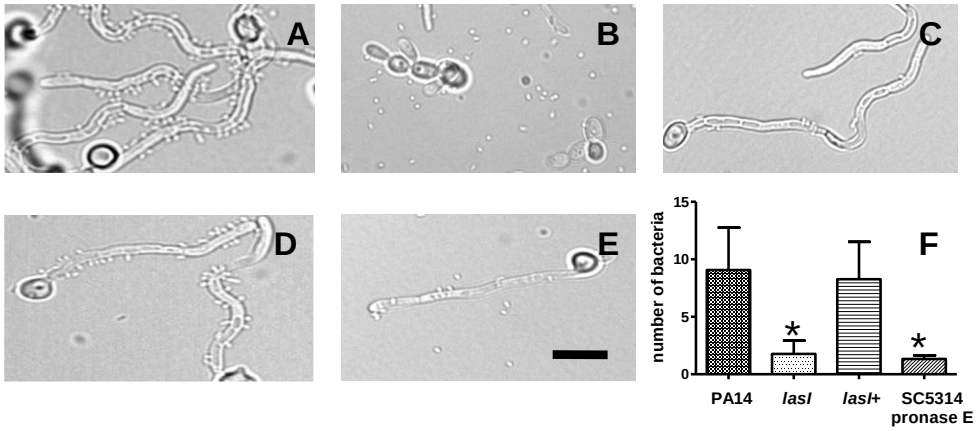

Figure 2 Bright field microscopic images and quantitative enumeration of the interaction between *C. albicans* with *P. aeruginosa* strains.
(A) *C. albicans* SC5314 hyphae with *P. aeruginosa* PA14.
(B) *C. albicans* SC5314 yeast with *P. aeruginosa* PA14.
(C) *C. albicans* SC5314 hyphae with *P. aeruginosa* PA14 *lasI*.
(D) *C. albicans* SC5314 hyphae with *P. aeruginosa* PA14 *lasI*, grown in the presence of 3OC12HSL.
(E) pronase E treated *C. albicans* SC5314 hyphae with *P. aeruginosa* PA14.
Scale bar corresponds with 10 µm.
(F) Average number of *P. aeruginosa* adhering per 10 µm hyphal length for *C. albicans* SC5314. *lasI+* indicates *P. aeruginosa* PA14 *lasI* grown in the presence of $3OC_{12}HSL$. Enumeration for pronase E treated *C. albicans* SC5314 hyphae cannot be done due to its loose adhesion to the well plate surface. Error bars represent SD over four experiments with separately cultured organisms and involving 30 hyphae per bacterium-fungus pair. *Statistically significant differences ($P < 0.05$; Student's t-test) with respect to the pairs *C. albicans* SC5314 - *P. aeruginosa* PA14.

Differences in LW surface free energy components were minor. All hydrophilic cell surfaces had a relatively large electron-donating surface free energy parameter γ^- (46.9 to 68.0 mJ/m2), in addition to a small electron-accepting surface free energy

parameter γ^+ (maximally 1.3 mJ/m2). The hyphal surface had a zero electron-donating surface free energy parameter with a small electron-accepting parameter (4.5 mJ/m2). In contrast, the pronase E treated hyphal cell surface showed a sizeable (58.1 mJ/m2) electron-donating surface free energy parameter in the absence of an electron-accepting surface free energy parameter.

Table 1 Contact angles and resulting surface free energies of the different *P. aeruginosa* and *C. albicans* strains used in this study. The data represents the average ± SD over three separately grown cultures. Three different lawns were prepared from each culture and on each lawn one droplet of each liquid was placed.

	θ_w	θ_F	θ_M	θ_B	γ^-	γ^+	γ^{LW}
P. aeruginosa	(degrees)				(mJ/m^2)		
PA14	29 ± 5	46 ± 6	66 ± 13	49 ± 5	68.0	0.4	27.8
PA14 *lasI*	27 ± 7	43 ± 5	66 ± 9	43 ± 3	66.0	0.5	29.0
PA14 *lasI* + $3OC_{12}HSL$	31 ± 6	41 ± 5	65 ± 7	45 ± 5	56.6	0.9	29.0
C. albicans							
yeast	25 ± 7	25 ± 2	55 ± 2	23 ± 4	46.9	1.3	36.1
hyphae	107 ± 9	51 ± 3	49 ± 4	28 ± 5	0.0	4.5	37.1
pronase E treated hyphae	34 ± 6	46 ± 6	43 ± 3	30 ± 4	58.1	0.0	38.4

The bacterial and fungal cell surface free energies listed in Table 1 were subsequently used to calculate interfacial free energies of adhesion for all combinations of *P. aeruginosa* and *C. albicans* (Table 2). The LW component was always favorable ($\Delta G^{LW}_{adh} < 0$) toward adhesion, but the AB interaction energies differed considerably depending on the *C. albicans* morphology considered. *P. aeruginosa* strains experienced unfavorable ($\Delta G^{AB}_{adh} > 0$) AB conditions for

adhesion to yeast cells (35.3 to 40.6 mJ/m2) and pronase E treated (46.1 to 55.0 mJ/m2) hyphae, in line with experimental observations. However, all *P. aeruginosa* strains showed attractive AB interactions for adhesion to hyphae (-26.9 to -27.3 mJ/m2), despite the fact that *P. aeruginosa* PA14 *lasI* hardly adhered to hyphae. This suggests that the mannoprotein layer on the surface of hyphae that is removed by pronase E treatment, is responsible for creating favorable AB conditions allowing adhesion of *P. aeruginosa* with *C. albicans*.

Table 2 LW and AB components of the interfacial free energy of adhesion (ΔG_{adh}) between different pairs of bacteria and fungi.

		P. aeruginosa		
C. albicans	ΔG_{adh} (mJ/m^2)	PA14	PA14 *lasI*	PA14 *lasI* + $3OC_{12}HSL$
yeast	LW	-1.6	-2.0	-1.9
	AB	**40.6**	**39.9**	**35.3**
SC5314 hyphae	LW	-1.7	-2.1	-2.0
	AB	**-27.3**	**-26.9**	**-27.2**
pronase E treated hyphae	LW	-1.8	-2.2	-2.2
	AB	**55.0**	**53.4**	**46.1**

Interestingly, AFM adhesion forces between *P. aeruginosa* strains and *C. albicans* hyphae became stronger with time in an exponential fashion (see Figure 3 for examples), while they remained negligible at all times with the yeast morphology. Bond-maturation increased the adhesion forces with hyphae by a factor of 2 to 4, but not for *P. aeruginosa* PA14 *lasI* (see Figure 3). Bond-maturation for *P. aeruginosa* PA14 occurred with a characteristic time constant, τ, of 5 s. Bond-maturation of *P. aeruginosa* PA14 *lasI* grown in the presence of $3OC_{12}HSL$ followed a similar pattern (τ equals 14 s).

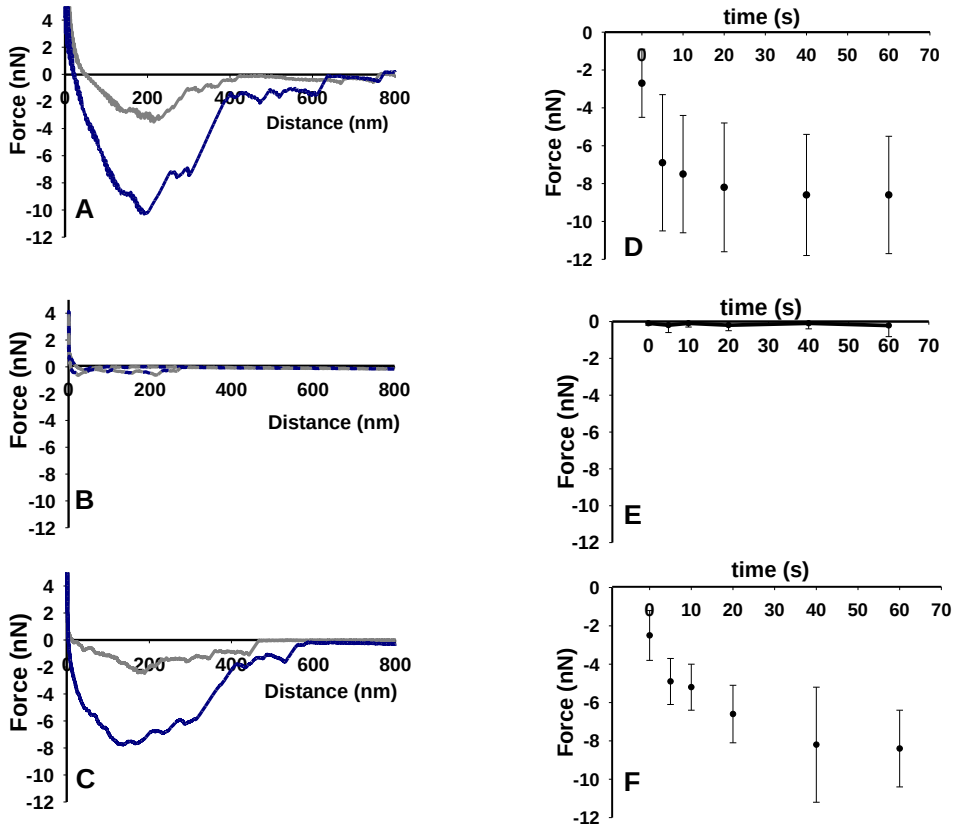

Figure 3 Force-distance curves between different bacterium-fungus pairs. Example curves upon initial contact (grey lines) and after 60 s (blue lines) bond-maturation are shown (left panels). Adhesion forces as a function of the maturation time for the interaction between each bacterium-fungus pair are shown in the right panels. Median and error bars, denoting the interquartile ranges, are derived from at least 60 force-distance curves, taken from three independent cultures.
(A, D) *C. albicans* hyphae and *P. aeruginosa* PA14.
(B, E) *C. albicans* hyphae and *P. aeruginosa* PA14 *lasI*.
(C, F) *C. albicans* hyphae and *P. aeruginosa* PA14 *lasI* grown in the presence of $3OC_{12}HSL$.

Bond-maturation enables calculation of an initial adhesion force and an adhesion force after bond-maturation. Major differences existed in AFM force-distance curves recorded both prior to and after bond-maturation between the different

P. aeruginosa strains and *C. albicans* SC5314 hyphae, as summarized in Figure 4A. The strongest adhesion forces were observed for *P. aeruginosa* PA14 (-8.6 nN) and *P. aeruginosa* PA14 *lasI* grown in the presence of $3OC_{12}HSL$ (-8.4 nN). In contrast, adhesion forces of *P. aeruginosa* PA14 *lasI* with hyphae were virtually absent (-0.4 nN), indicating that strong adhesion forces of *P. aeruginosa* PA14 *lasI* can be restored by growth in the presence of the synthetic $3OC_{12}HSL$ (Figure 4A).

AFM measurements showed a drastic reduction in adhesion forces recorded between *P. aeruginosa* PA14, *P. aeruginosa lasI* and *P. aeruginosa* PA14 *lasI* grown in the presence of $3OC_{12}HSL$ and yeast cells of *C. albicans* SC5314 (Figure 4B), which is in line with the observed absence of adhesion illustrated in Figure 2. Removal of the outer mannoprotein layer from the hyphal surface by pronase E treatment reduced the interaction with *P. aeruginosa* PA14 as well (Figure 4C). The role of AB interactions in hypha-bacterium interaction can also be derived by the development of minor adhesion peaks in the retract flanks of the AFM force-distance curves. These minor adhesion peaks become more distinct after bond-maturation and can be analyzed to separate the AB contribution of a single-bond ($f_{AB\text{-}bonding}$) from the overall, non-specific force ($F_{NS\text{-}bonding}$), as done in Figure 5 and summarized in Table 3 for the interaction of the most strongly adhering *P. aeruginosa* PA14 with untreated and pronase E treated hyphae. Poisson analysis confirms the conclusion drawn from surface thermodynamics that adhesion between *P. aeruginosa* PA14 with *C. albicans* hyphae is governed by an interplay of non-specific (LW) and AB forces. Moreover, the values of single AB bonds derived from Poisson analysis vary qualitatively in line with ΔG^{AB}_{adh}, although not becoming repulsive for the interaction between *P. aeruginosa* PA14 with *C. albicans* pronase E treated hyphae, as suggested by surface thermodynamics. Note that $f_{AB\text{-}bonding}$ cannot be directly compared with $F_{NS\text{-}bonding}$, since it denotes the magnitude of a discrete single-bond, whereas $F_{NS\text{-}bonding}$

describes the overall, non-specific force. The differences in $f_{AB\text{-}bonding}$ in Table 3 over the different pairs indicate that different molecular moieties are involved in their adhesion.

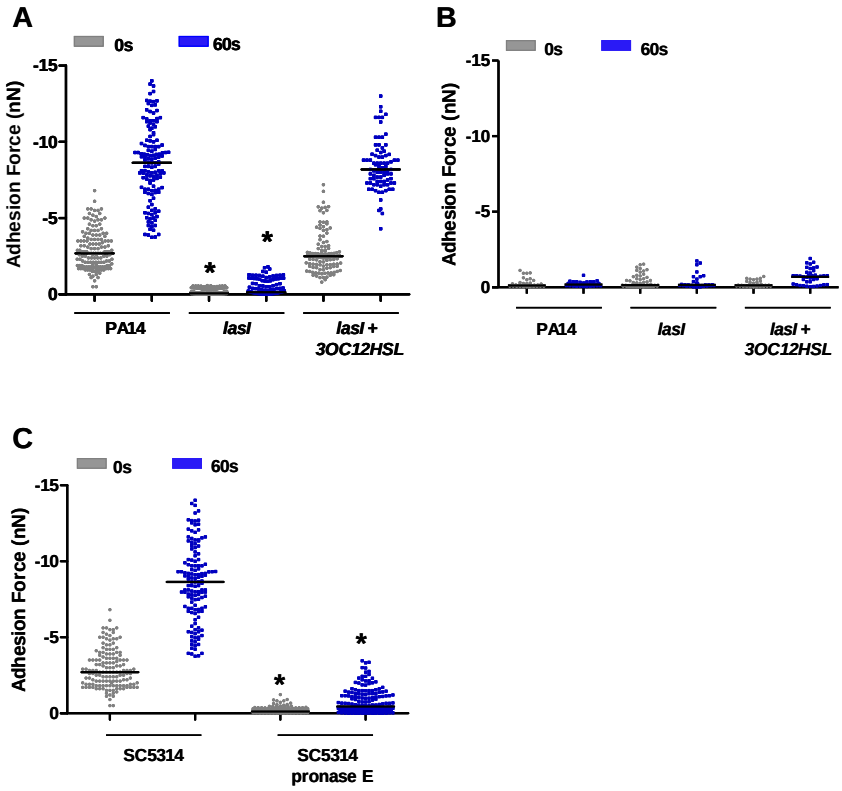

Figure 4 Vertical scatter bars of adhesion forces between different bacterium-fungus pairs. Adhesion forces were measured immediately upon contact (0 s) and after bond-maturation (60 s). Each data point corresponds to a single force-distance curve recorded between a bacterium and a hypha.
(A) *C. albicans* SC5314 hyphae with *P. aeruginosa* PA14 and *lasI* mutant
(B) *C. albicans* SC5314 yeast with *P. aeruginosa* PA14 and *lasI* mutant
(C) *C. albicans* SC5314 hyphae prior to and after pronase E treatment with *P. aeruginosa* PA14 .
Median force values are indicated with a line. *Statistically significant differences ($P < 0.05$; Mann-Whitney test) with respect to the pairs *C. albicans* SC5314 - *P. aeruginosa* PA14.

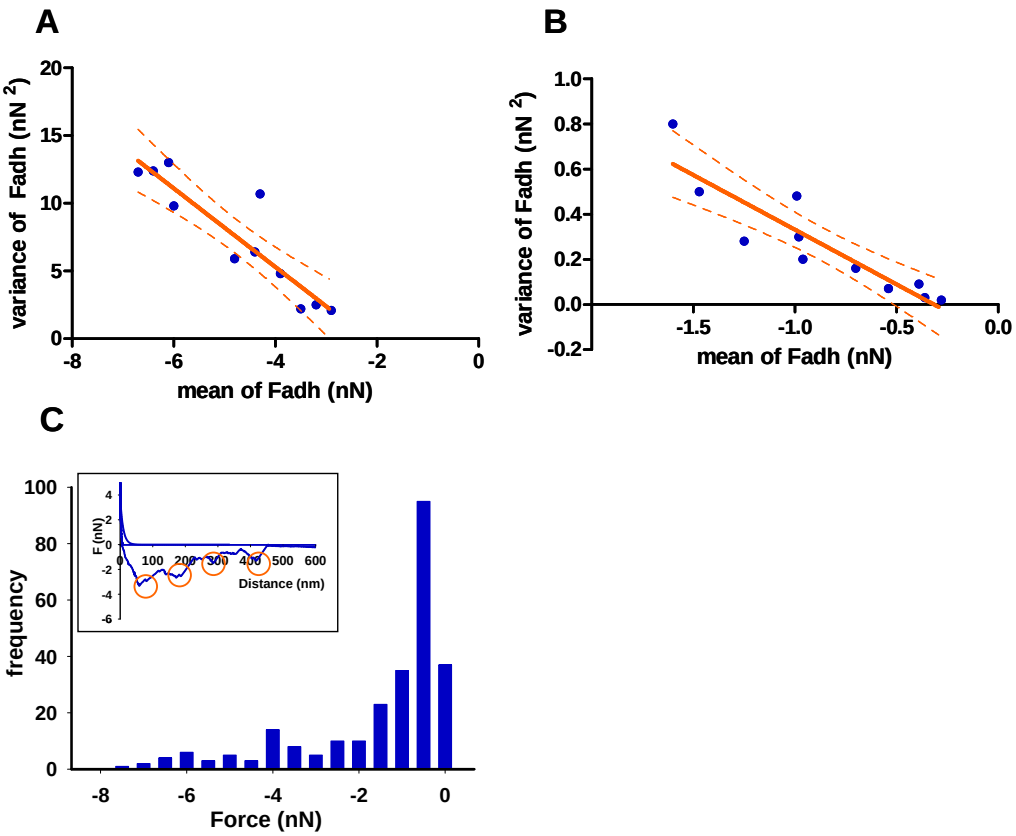

Figure 5 Poisson analysis of the retract force-distance curves recorded after 60 s of bond-maturation to decouple the single-bond acid-base contribution and the overall, non-specific force to the total adhesion force between *P. aeruginosa* PA14 and hyphae of

(A) *C. albicans* SC5314,

(B) *C. albicans* SC5314 after pronase E treatment,

The acid-base contribution can be taken directly from the slope of a straight line through the data points, while the intercept with the mean adhesion force axis represents the overall non-specific force. Each data point corresponds to a single bacterium-fungus pair. Dashed orange lines represent 95% confidential intervals.

(C) an example of a retract force-distance curve obtained after 60 s of bond-maturation between *P. aeruginosa* PA14 and *C. albicans* hyphae with the orange circles indicating the major and minor adhesion peaks. Adhesion forces from each force-distance curve follow a Poisson distribution, as indicated.

Table 3 Single-bond, acid-base force contributions ($f_{AB\text{-}bonding}$) and overall non-specific forces ($F_{NS\text{-}bonding}$) involved in adhesion between *P. aeruginosa* PA14 and different hyphae. Values are derived from Poisson analysis of the retract force-distance curves taken after 60 s of bond-maturation (n = 60 for each value).

Bacterial-fungal pair	$f_{AB\text{-}bonding}$ (nN)	$F_{NS\text{-}bonding}$ (nN)
PA14 - SC5314 hyphae	-2.9	-2.2
PA14 - pronase E treated hyphae	-0.5	-0.3

DISCUSSION

P. aeruginosa is a highly virulent bacterium that expresses a plethora of surface-associated or secreted virulence factors [23, 32]. Because of its lethality, many species have developed early warning systems to detect and evade *P. aeruginosa* [33]. *C. albicans* can sense the presence of *P. aeruginosa* by detecting its QS molecule $3OC_{12}HSL$ and responds by changing its mode of growth from the hyphal to the adhesion-resistant yeast morphology [24].

Using a combination of surface thermodynamics and Poisson analysis of AFM adhesion forces, it was shown in this study that the outermost mannoprotein layer on the hyphal surface serves to create favorable conditions for interaction through attractive LW and AB forces, allowing *P. aeruginosa* to closely approach the hyphal surface. Favorable overall attraction however, is not a sufficient condition for adhesion of *P. aeruginosa* to hyphal surfaces and strong stereo-chemical interactions between hyphal cell wall and specific binding proteins on the bacterial cell surface are required for adhesion. Furthermore bacteria - hyphae interaction is regulated by QS since the measured adhesion forces were significantly decreased for the *P. aeruginosa* PA14 *lasI* mutant but were restored to wild-type levels cued by addition of synthetic $3OC_{12}HSL$.

The merits of surface thermodynamics have often been questioned with respect to offering a mechanism for microbial adhesion [34]. One of most heard

criticisms involves the lack of sensitivity of macroscopic contact angles for the presence of localized, sparsely present stereo-chemical, receptor-ligand sites. On the other hand, due to the extreme surface sensitivity of contact angles for overall chemical changes, they strongly reflect the outermost properties of a cell surface and small compositional differences in the outer fungal mannoprotein layer [19] as well as differences in the extend of O-glycosylation between yeast and hyphal morphologies, have a major impact on contact angles and cell surface free energies. Therewith surface thermodynamics only partially explains why *P. aeruginosa* strains adhere or do not adhere to *C. albicans*. We can speculate that in the presence of favorable thermodynamic conditions, certain components of the hyphal but not of the yeast cell wall serve as ligands for bacterial binding proteins allowing adhesion and subsequent killing to occur. Brand and coworkers illustrated that O-glycosylation, and specifically O-mannosylation of cell-wall proteins induced killing of hyphae by *P. aeruginosa* [17]. Possibly, increased killing of hyphae lacking O-glycosylation is due to increased presence of specific sites on hyphal cell wall recognized by binding proteins of *P. aeruginosa*.

It is clear however, that in the absence of attractive AB interactions, close approach between the bacterial and fungal cell surfaces is impossible and specific stereo-chemical interactions [35] cannot occur. AFM, as applied here, puts the conclusions drawn from surface thermodynamics regarding the role of acid-base interaction on a firm basis. The first major peak in the force-distance curve is around 200 nm from the surface, while loss of physical interaction occurs around 600 nm from the surface. Physico-chemically, this is far beyond the range of the attractive AB or LW forces, indicating that surface appendages on the interacting surfaces are stretched upon retract till bond rupture occurs [12]. Since electron microscopic analysis has pointed out that the cell wall of *C. albicans* is around 200 nm thick, with an electron dense outer surface representing mannoproteins [36]

stretching up to a distance of 600 nm seems feasible, especially considering that surface appendages on the *P. aeruginosa* surface can also stretch. Removal of the outer mannoprotein layer on *C. albicans* using pronase E, decreased the rupture distance to around 220 nm, demonstrating that the rupture distance is not only determined by surface appendages on the *P. aeruginosa* cell surface but also by stretching of mannoproteins on the *C. albicans* surface.

In conclusion, the mannoprotein layer on the hyphal surface serves to create favorable interaction conditions through attractive LW and AB forces, allowing *P. aeruginosa* to closely approach the hyphal surface. Favorable overall attraction however, is not a sufficient condition for adhesion of *P. aeruginosa* to hyphal surfaces. Expression of specific binding proteins by *P. aeruginosa* that interacts stereo-chemically with the fungal cell wall is required for final adhesion to take place. Expression of binding proteins by *P. aeruginosa* is regulated by QS, which at the same time warns *C. albicans* against its presence and stimulates the fungus to adapt its yeast morphology, exhibiting unfavorable AB conditions and lacking strong adhesion forces with *P. aeruginosa*.

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Chapter 3

Surface thermodynamic and adhesion force evaluation of the role of chitin-binding protein in the physical interaction between *Pseudomonas aeruginosa* and *Candida albicans*

(Ovchinnikova E.S., Krom B.P., Harapanahalli A.K.,Busscher H.J., Van der Mei H.C., submitted to *Langmuir*)

ABSTRACT

Candida albicans and *Pseudomonas aeruginosa* are able to form pathogenic polymicrobial communities. *P. aeruginosa* colonizes and kills hyphae, but is unable to attach to yeast. It is unknown why the interaction of *P. aeruginosa* is different with yeast than with hyphae. Here we aim to evaluate the role of *P. aeruginosa* chitin-binding protein (CbpD) in its physical interaction with *C. albicans* hyphae or yeast, based on surface thermodynamic and atomic force microscopic analyses. A *P. aeruginosa* mutant lacking CbpD was unable to express strong adhesion forces with hyphae (-2.9 nN) as compared with the parent strain *P. aeruginosa* PAO1 (-4.8 nN) and showed less adhesion to hyphae. Also blocking of CbpD using N-acetyl-glucosamine yielded a lower adhesion force (-4.3 nN) with hyphae. Strong adhesion forces were restored after complementing the expression of CbpD in *P. aeruginosa* PAO1 *cbpD* yielding an adhesion force of -5.1 nN. Regardless of the absence or presence of CbpD on the bacterial cell surfaces, or their blocking, *P. aeruginosa* experienced favorable thermodynamic conditions for adhesion with hyphae, which were absent with yeast. In addition, adhesion forces with yeast were less than 0.5 nN in all cases. Concluding, CbpD in *P. aeruginosa* is responsible for strong physical interactions with *C. albicans* hyphae. The development of this interaction requires time due to the fact that CbpDs have to invade the outermost mannoprotein layer on the hyphal cell surfaces. In order to do this, thermodynamic conditions at the outermost cell surfaces have to be favorable.

INTRODUCTION

In nature, microorganisms often exist within polymicrobial communities. Moreover, humans can also be co-infected with multiple pathogens, and these polymicrobial infections have properties that are distinct from single-species infections [1, 2]. *Candida albicans* is a polymorphic human fungal pathogen that can cause common mucosal infections as well as serious life-threatening systemic infections in immuno-compromised patients suffering from HIV-infection or tissue transplantation [3]. *C. albicans* can exist in a yeast morphology or as filamentous cells that can be either hyphae or pseudohyphae [4-6]. The ability to transit from the yeast to hyphal form is a crucial virulence determinant toward mammals [5].

C. albicans and *Pseudomonas aeruginosa* are able to form a polymicrobial community, and can be co-isolated from patients with cystic fibrosis or severely burned patients [2, 7]. *In vivo* and *in vitro* studies demonstrate that *P. aeruginosa* readily attaches to and forms biofilms on the hyphal surface of *C. albicans* [8]. While *P. aeruginosa* quickly colonizes and kills hyphae, it is not able to attach to or form biofilms on yeast cells [8]. In order to avoid killing by *P. aeruginosa*, *C. albicans* changes its growth morphology from hyphae to yeast under the influence of 3-oxo-C_{12}-homoserine lactone, a low-molecular-weight quorum sensing molecule synthesized by *P. aeruginosa* [8].

It is still not well understood why the interaction of *P. aeruginosa* is so different with yeast than with hyphae, although differences in composition of cell wall proteins and carbohydrates of hyphae *versus* yeast have been demonstrated [9]. *P. aeruginosa* not only interacts with *C. albicans* hyphae but also with hyphae of *Aspergillus nidulans* and *Alternaria alternato*, which suggests that there is a common recognition site for *P. aeruginosa* on the different fungal surfaces [10].

P. aeruginosa expresses a potentially specific fungal receptor, the chitin-binding protein CbpD, which may be expected to interact with chitin, present in both the yeast and hyphal cell wall [11-13]. CbpD is present in many clinical

isolates of *P. aeruginosa* and it has been proposed that CbpD plays a role in *P. aeruginosa* pathogenicity by mediating attachment to chitin-containing substrates or interacting with N-glycosylated cell wall receptors containing *N*-acetyl-glucosamine residues in eukaryotic cells [12]. Fungal chitin however, is not necessarily exposed to the environment but probably mainly located at the inner side of the cell wall, covered by a mannoprotein layer, containing about 10% of the dry weight of both yeast cells and hyphae. The exact protein composition of the fungal cell wall mannoprotein layer can differ between yeast cells and hyphae [13] and previously we have shown that this mannoprotein layer dictates the thermodynamic conditions at the interface that do or do not allow *P. aeruginosa* to adhere to the fungal cell wall [14].

The thermodynamic conditions at an interface between two interacting surfaces can be calculated from contact angle measurements with different liquids on the interacting surfaces [15, 16]. Thermodynamically favorable conditions for adhesion are characterized by a negative interfacial free energy of adhesion (ΔG_{adh}), while unfavorable conditions are accompanied by positive values for (ΔG_{adh}). The long-range Lifshitz–Van der Waals component of the interfacial free energy of adhesion (ΔG_{adh}) is nearly always negative ($\Delta G_{adh}^{LW} < 0$) and responsible for long-range attraction. The short-range acid–base component of interfacial free energy of adhesion (ΔG_{adh}^{AB}) usually dominates the long-range Lifshitz–Van der Waals component and can either be positive (unfavorable conditions for adhesion) or negative (favorable conditions). Favorable acid-base conditions have been demonstrated to control the co-aggregation between *Streptococcus gordonii* NCTC 7869 and *C. albicans* ATCC 10261 [17]. However, favorable thermodynamic conditions at an interface are not always sufficient for strong adhesion forces between bacteria and fungi to develop [14] and it has been recently recognized [18] that surface thermodynamics merely dictate

whether or not the interfacial conditions between bacteria and cells are suitable for strong adhesion forces to develop. Atomic force microscopy (AFM) [19] can be used to directly measure the adhesion forces between different microorganisms. AFM records the adhesion forces as a function of the separation distance between a bacterial probe attached to a flexible cantilever and an organism immobilized on a substratum surface. Upon initial approach, a repulsive force is measured, but upon retract attractive forces are measured that decrease with increasing separation distance until rupture of the bond occurs [20, 21].

The aim of the present study is to evaluate the role of CbpD on *P. aeruginosa* cell surfaces in its physical interaction with *C. albicans* hyphae or yeast, based on a surface thermodynamic and atomic force microscopic analyses. To this end, we compared adhesion between *C. albicans* and *P. aeruginosa* PAO1 in absence and presence of adsorbed N-acetylglucosamine (the monomer of chitin) to block CbpD. In addition, a *P. aeruginosa* mutant without CbpD expression and its complemented derivative with restored expression of CbpD were used**.**

MATERIALS AND METHODS

Strains, growth conditions and harvesting

The fungus *C. albicans* SC5314 [22] and bacterial strains *P. aeruginosa* PAO1 [23] (wild type) and the mutant *P. aeruginosa* PAO1 *cbpD* [24], lacking CbpD were used. Chitin-binding protein expression in the *cbpD* mutant was restored by introducing pJF38 [11] using electroporation [25]. Transformants were selected on tryptone soy broth with 1.5% bactoagar (TSB, Oxoid, Basingstoke, UK) agar plates containing 100 µg/ml of ampicillin and presence of the plasmid was checked by agarose gel electrophoresis. Strains were grown on TSB agar plates and single colonies were used to inoculate 5 ml TSB for bacterial precultures or 5 ml yeast nitrogen base without amino acids *(*YNB; Difco, Sparks, MD, USA) pH 7, containing

0.5% D-glucose for *C. albicans* precultures [26]. The antibiotic concentrations used for selection and maintenance of mutants were 25 µg/ml of kanamycin for *P. aeruginosa* PAO1 *cbpD*, and 100 µg/ml of ampicillin and 25 µg/ml of kanamycin for *P. aeruginosa* PAO1 *cbpD* + pJF38. Precultures of *C. albicans* and bacteria were grown at 30°C and 37°C respectively, for 24 h with rotation (150 rpm) and used to inoculate a main culture (1:50 dilution). Main bacterial cultures were grown for an additional 18 h under the same conditions. *C. albicans* germ-tubes, representing young hyphae, were induced by growing a culture (1:50 dilution) in YNB pH 7.0 for 4 h with rotation (150 rpm) at 37°C [27]. Germ-tube formation was obtained at 90-95% efficiency under these conditions, as microscopically confirmed. Main cultures were harvested by centrifugation for 5 min at 6,250 x g and 14,800 x g for *P. aeruginosa* and *C. albicans*, respectively followed by two washes with ultrapure water and resuspended in demineralized water.

Adhesion assay

Actual adhesion of *P. aeruginosa* to *C. albicans* in either the yeast or hyphal morphology was enumerated per unit hyphal length using bright field microscopy. Hyphal formation was induced by growth at 37°C in 12 wells plate (Costar, Corning Inc., NY, USA). In order to adhere hyphae to the surface of the well we made 1:100 dilution of *C. albicans* pre-culture in 1 ml of YNB, pH 7.0 supplemented with 0.5% D-glucose and incubated it without shaking at 37°C. After 4 h of growth, wells were washed once and 1 ml of bacterial suspension in 10 mM potassium phosphate buffer, pH 7 at an OD_{600} of 0.1 was added. After 90 min of co-incubation at 37°C under static conditions, wells were gently washed twice with phosphate buffer and images were captured at five randomly chosen locations in the wells using a 40x water immersion objective. The adhesion assay was also carried out with *P. aeruginosa* PAO1 after adsorption of N-

acetylglucosamine (GlcNAc, the monomer of chitin), to demonstrate the effects of blocking possible chitin-binding sites. To this end, GlcNAc was allowed to adsorb to *P. aeruginosa* PAO1 for 30 min from a 2 mg/ml solution in potassium phosphate buffer, washed once with PBS to remove the excess GlcNAc after which the strain was used in the adhesion assay as described above.

Contact angle measurements and surface thermodynamic analysis

To determine microbial cell surface free energies, microorganisms were layered onto 0.45 µm or 3 µm pore-size Millipore filters for bacteria and yeast, respectively using negative pressure. Bacteria were resuspended in demineralized water, while fungi were layered from a suspension in TNMC buffer (1 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$). The filters were left to dry in ambient air, until stable, so-called plateau water contact angles of sessile droplets [28] could be measured, representing a state of cell surface hydration most closely resembling the physiologically occurring one [29]. For each strain, three independently grown cultures were used, while three microbial lawns were prepared out of each culture.

To allow surface thermodynamic analysis [29], contact angles were also measured with formamide, methylene iodide and 1-bromonaphthalene and the microbial cell surface free energy (γ^{tot}), its LW (γ^{LW}) and AB (γ^{AB}) components and the electron-donating and accepting parameters [30] were calculated from the measured contact angles. The bacterial and fungal surface free energies were subsequently employed to calculate the free energy of adhesion (ΔG_{adh}) between the bacterial (B) and fungal cell-surface (F) in an aqueous suspension (L) and its LW ($\Delta G_{adh}{}^{LW}$) and AB ($\Delta G_{adh}{}^{AB}$) components

$$\Delta G_{adh} = \Delta G_{adh}^{LW} + \Delta G_{adh}^{AB} \quad (1)$$

where

$$\Delta G_{adh}^{LW} = \left(\sqrt{\gamma_B^{LW}} - \sqrt{\gamma_F^{LW}}\right)^2 - \left(\sqrt{\gamma_B^{LW}} - \sqrt{\gamma_L^{LW}}\right)^2 - \left(\sqrt{\gamma_F^{LW}} - \sqrt{\gamma_L^{LW}}\right)^2 \quad (2)$$

and

$$\Delta G_{adh}^{AB} = 2\left[\sqrt{\gamma_L^+}\left(\sqrt{\gamma_B^-} + \sqrt{\gamma_F^-} - \sqrt{\gamma_L^-}\right) + \sqrt{\gamma_L^-}\left(\sqrt{\gamma_B^+} + \sqrt{\gamma_F^+} - \sqrt{\gamma_L^+}\right) - \sqrt{\gamma_B^-\gamma_F^+} - \sqrt{\gamma_B^+\gamma_F^-}\right] \quad (3)$$

According to surface thermodynamics, adhesion will be favorable if $\Delta G_{adh} < 0$ [15].

Atomic force microscopy

C. albicans SC5314 was immobilized on glass slides (Menzel, GmbH, Braunschweig, Germany), coated with positively charged poly-L-lysine [31]. A fungal suspension in demineralized water was deposited onto the coated glass and left to settle at room temperature for 20 min. Non-adhering cells were removed by rinsing with demineralized water and the slide was kept hydrated prior to AFM analysis in phosphate buffer. To create a bacterial probe, *P. aeruginosa* was immobilized onto poly-L-lysine treated tipless "V"-shaped cantilevers (DNP-0,Veeco Instruments Inc., Woodbury, NY, USA). To this end, cantilevers were mounted in a micromanipulator under microscopic observation to allow only the tip of the cantilever to be coated. A droplet of poly-L-lysine solution was placed on a glass slide, and the tip of the cantilever was dipped in the droplet for 1 min. After air-drying the cantilever for 2 min, it was dipped in a bacterial suspension for 1 min. Bacterial probes were freshly prepared for each experiment. In order to verify that a bacterial probe enabled a single contact with the fungal surface, a scanned

image was made at the onset of each experiment and examined for double contour lines, which would be indicative of multiple bacteria contacting the fungal surface. Any probe exhibiting double contour lines was discarded. At this point it must be noted however, that double contour line images seldom or never occurred, since it represents the unlikely situation that bacteria on the cantilever are equidistant to an immobilized fungal surface within the small range of the interaction forces, which is unlikely if only by the angle under which the cantilever is in contact with the substratum.

Adhesion forces were measured at room temperature in phosphate buffer, using an optical lever microscope (Nanoscope IV, Digital Instruments, Veeco Instruments Inc.). For each bacterial probe, force curves were measured after different bond-maturation times up to 60 s on the same, randomly chosen spot on a fungal cell with z-scan rates of less than 1 Hz. To ensure that no bacteria detached from the cantilever during the experiment, control force-distance curves were made with 0 s contact time after each set of measurements. Whenever the "0 s contact time" forces measured deviated more than 0.5 nN from the initial measurement, a bacterial probe was considered damaged and replaced. For each combination of a bacterial strain and fungal–coated glass surface, five different probes were employed on average and the number of bacterial probes used depended on the outcome of the control measurements. Calibration of each cantilever was done using the thermal tuning method (Nanoscope V6.13r1), yielding a range of spring constants from 0.04 to 0.1 (N m^{-1}).

For each bacterial probe, the maximum adhesion forces were plotted as a function of the bond-maturation time Δt, and fitted to

$$F(\Delta t) = F_{0s} + (F_{\infty} - F_{0s})(1 - \exp\{-\Delta t/\tau\}) \quad (4)$$

with F_{0s} being the maximum adhesion force at 0 s contact time, F_{∞} being the maximum adhesion force after bond-maturation, and τ being the characteristic time needed for the adhesion force to strengthen [32].

Statistics

Adhesion forces for different fungus-bacterium pairs were compared using non-parametric analyses (Mann-Whitney test). Differences were considered significant when $P < 0.05$.

RESULTS

Microscopic enumeration showed that *P. aeruginosa* PAO1 adhered to hyphae of *C. albicans* SC5314, but not to yeast cells (see Figure 1). The PAO1 strain lacking CbpD, adhered to hyphae in significantly lower numbers, as did *P. aeruginosa* PAO1 after adsorption of GlcNAc to block adhesion sites, as can be seen from the quantitative presentation of the data in Figure 1F. Restoration of CbpD expression in *P. aeruginosa* PAO1 *ΔcbpD* + pJF38 restored their adhesion to hyphae to the level of the parent strain (see Figure 1).

AFM adhesion forces between *P. aeruginosa* strains and *C. albicans* hyphae became stronger with time in an exponential fashion (see Figure 2 for examples). Bond-maturation for *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1 *cbpD* lacking CbpD or *P. aeruginosa* PAO1 after blocking CbpD with GlcNAc occurred with a characteristic time constant, τ, of 25, 33 and 35 s, respectively. Interestingly, strong adhesion forces were observed again for *P. aeruginosa* PAO1 *cbpD* + pJF38 in which CbpD expression was restored, and bond-maturation occurred with a relatively short characteristic time constant, τ, of around 13 s.

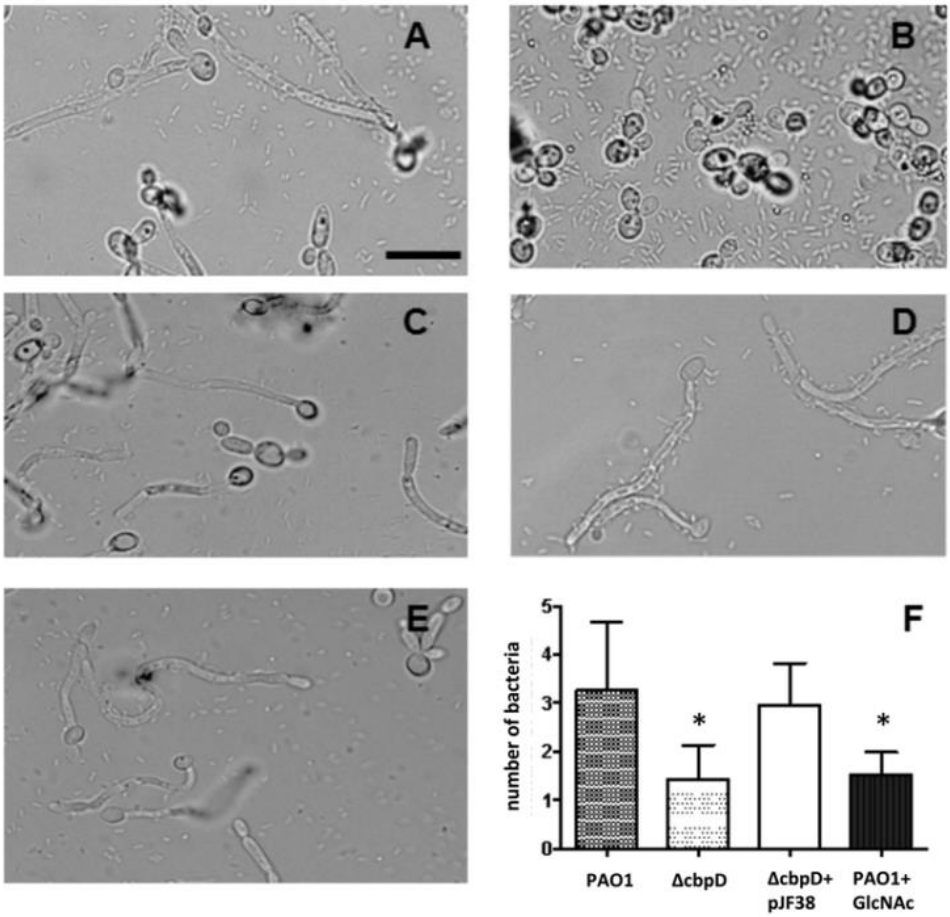

Figure 1 Bright field microscopic images and quantitative enumeration of the interaction between *C. albicans* SC5314 with different *P. aeruginosa* mutants (scale bar corresponds with 10 µm).
(A) *C. albicans* hyphae with *P. aeruginosa* PAO1
(B) *C. albicans* yeast with *P. aeruginosa* PAO1
(C) *C. albicans* hyphae with *P. aeruginosa cbpD*
(D) *C. albicans* hyphae with *P. aeruginosa cbpD* + pJF38
(E) *C. albicans* hyphae with *P. aeruginosa* PAO1 pre-incubated with 2 mg/ml of N-acetylglucosamine.
(F) average number of *P. aeruginosa* adhering per 10 µm hyphal length. Error bars represent SD over four experiments with separately cultured organisms and involving 30 hyphae per bacterium-fungus pair. *Statistically significant differences ($P < 0.05$; Student's t-test) with respect to the pairs *C. albicans* SC5314 - *P. aeruginosa*.

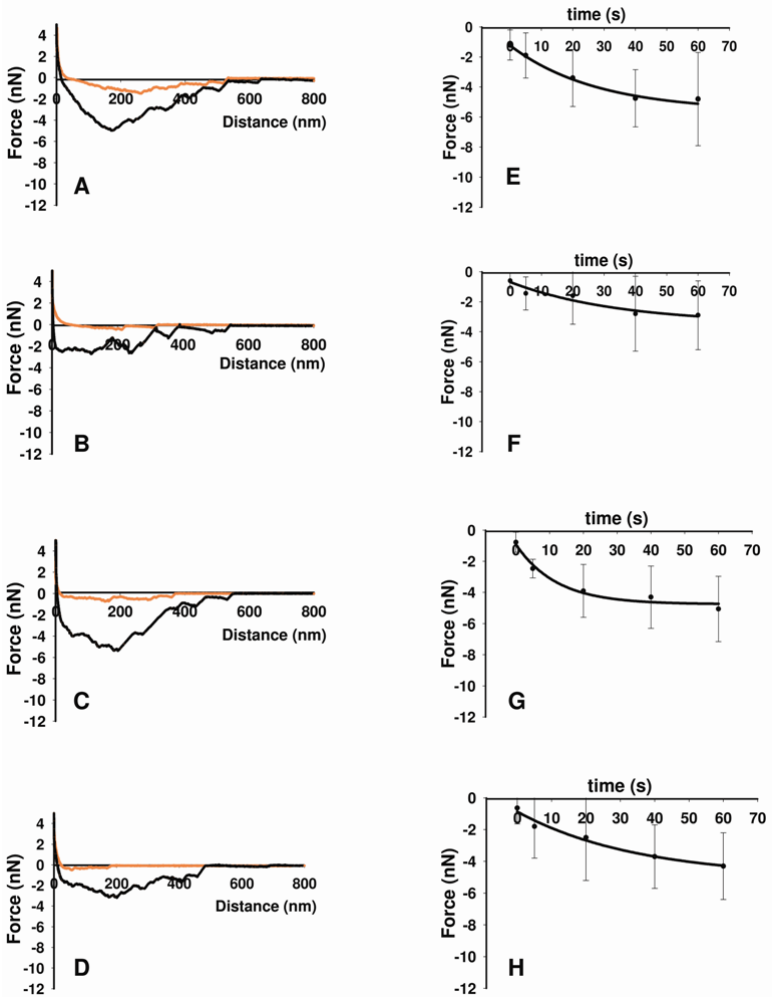

Figure 2 Examples of force-distance curves between different bacterium - *C. albicans* SC5314 pairs. Example curves upon initial contact (orange) and after 60 s (black) bond-maturation are shown in the left panel, while median adhesion forces as a function of the bond-maturation time for each bacterium-fungus pair are shown in the right panels.
(**A**, **E**) *C. albicans* hyphae and *P. aeruginosa* PAO1.
(**B**, **F**) *C. albicans* hyphae and *P. aeruginosa cbpD*.
(**C**, **G**) *C. albicans* hyphae and *P. aeruginosa cbpD* + pJF38
(**D**, **H**) *C. albicans* hyphae and *P. aeruginosa* PAO1 + GlcNAc
Median and error bars, denoting the interquartile ranges, are derived from at least 60 force-distance curves, taken from three independent cultures.

Bond-maturation enables the calculation of an initial adhesion force and an adhesion force after bond-maturation, as summarized in Figure 3. The strongest adhesion forces after bond-maturation were observed for *P. aeruginosa* PAO1 (-4.8 nN) and *P. aeruginosa* PAO1 *cbpD* + pJF38 in which CbpD expression was restored (-5.1 nN). Significantly reduced adhesion forces were observed for *P. aeruginosa* PAO1 *cbpD* (-2.9 nN), lacking the CbpD and after blocking CbpD with GlcNAc in *P. aeruginosa* PAO1 (-4.3 nN), regardless of whether initial adhesion forces or forces after bond-maturation are considered. Interestingly, in case of blocking CbpD of *P. aeruginosa* PAO1 with GlcNAc, the spreading of measured adhesion forces is significantly larger than for the other *P. aeruginosa* strains, possibly due to incomplete coverage of CbpD by GlcNAc. This can be due to the

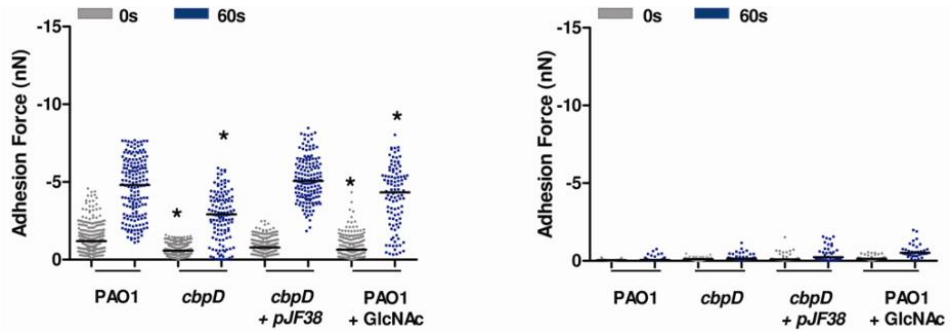

Figure 3 Vertical scatter bars of adhesion forces between different bacterium-*C. albicans* SC5314 pairs.
(A): Adhesion forces prior to and after 60 s bond-maturation between *C. albicans* hyphae with different *P. aeruginosa* PAO1 mutants and after adsorption of N-acetylglucosamine (GlcNAc).
(B): Same as above, for *C. albicans* yeast.
Each point represents the force recorded in an individual force-distance curve.
Median force values are indicated with a line. Statistically significant differences ($P < 0.05$; Mann-Whitney test) are indicated by an asterisk.

the fact that GlcNAc is also a key component of the peptidoglycan layer in the bacteria cell wall and therewith it can also block other sites on the bacterial surface than only CbpD. In contrast, adhesion forces of *P. aeruginosa* PAO1 with

yeast were virtually absent (-0.4 nN). Irrespective of the *P. aeruginosa* mutant used, the measured adhesion forces remained lower than 1 nN after 0 s surface delay time (see also Figure 3B) and even increased surface delay times up to 60 s did not result in a development of a significant adhesion force between *P. aeruginosa* and *C. albicans* in the yeast morphology.

Table 1 Contact angles and Lifshitz–Van der Waals (γ^{LW}) component and electron-donating (γ^{-}) and electron-accepting (γ^{+}) surface free energy parameters of the different *P. aeruginosa* mutants and both *C. albicans* SC5314 morphologies used in this study. The data represent the averages ± SD over three separately grown cultures. Three different lawns were prepared from each culture and on each lawn one droplet of each liquid was placed.

	θ_w	θ_F	θ_M	θ_B	γ^-	γ^+	γ^{LW}
P. aeruginosa	(degrees)				(mJ/ m^2)		
PAO1	27 ± 2	54 ± 4	53 ± 4	32 ± 2	78.1	0.0	35.2
cbpD	27 ± 3	60 ± 5	59 ± 6	30 ± 3	87.6	0.0	33.9
cbpD + pJF38	25 ± 2	40 ± 5	57 ± 8	33 ± 2	62.3	0.2	33.9
PAO1 + GlcNAc	25 ± 3	48 ± 5	58 ± 6	30 ± 3	72.0	0.0	34.5
C. albicans							
yeast	25 ± 7	25 ± 2	55 ± 2	23 ± 4	46.9	1.3	36.1
hyphae	107 ± 9	51 ± 3	49 ± 4	28 ± 5	0.0	4.5	37.1

*Contact angles taken from reference 14.

Contact angle measurements with liquids of different polarities were performed and surface free energy calculated (Table 1). According to the water contact angle, all cell surfaces were hydrophilic, with the exception of *C. albicans* hyphae, which were hydrophobic. Subsequently, free energies of bacterial

adhesion towards hyphae or yeast were calculated, assuming interaction in an aqueous phase (Table 2). The Lifshitz–Van der Waals component of bacterial cell surface energy was comparable across all strains and always favorable ($\Delta G^{LW}_{adh} < 0$) toward adhesion, whereas the acid-base component changed considerably depending on the *C. albicans* morphology considered (Table 2). All *P. aeruginosa* strains showed unfavorable ($\Delta G^{AB}_{adh} > 0$) AB conditions for adhesion to yeast (39.3 to 54.0 mJ/m^2) and favorable ($\Delta G^{AB}_{adh} < 0$) AB conditions for adhesion towards hyphae (-25.6 to -30.9 mJ/m^2).

Table 2 LW and AB components of the interfacial free energy of adhesion (ΔG_{adh}) between the different *P. aeruginosa* mutants and both *C. albicans* SC5314 morphologies. Negative interfacial free energies indicate thermodynamically favorable conditions for adhesion and are presented in bold for the AB components. LW components are always negative.

		P. aeruginosa			
C. albicans	ΔG_{adh} (mJ/ m^2)	PAO1	*cbpD*	*cbpD* + pJF38	PAO1 + GlcNAc
yeast	LW	-3.4	-3.1	-3.1	-3.2
	AB	**49.9**	**54.0**	**39.3**	**45**
hyphae	LW	-3.6	-3.3	-3.3	-3.3
	AB	**-28.6**	**-25.6**	**-29.8**	**-30.9**

DISCUSSION

Here we have evaluated the role of *P. aeruginosa* chitin-binding protein (CbpD) in its physical interaction with *C. albicans* hyphae or yeast cell surfaces. The influence of chitin-binding in the physical interaction between *P. aeruginosa* and *C. albicans* was established using microscopic observations, surface thermodynamics and adhesion force evaluation. A *P. aeruginosa* mutant lacking CbpD was unable

to express strong adhesion forces with *C. albicans* hyphae and showed less adhesion, while also blocking of CbpD on the bacterial cell surface yielded low adhesivity with hyphae. Strong adhesion forces as measured by AFM and adhesion to hyphae were completely restored after complementing the expression of CbpD in *P. aeruginosa* PAO1 *cbpD.* Regardless of the absence or presence of CbpD on the bacterial cell surfaces, or their blocking, *P. aeruginosa* experienced favorable thermodynamic conditions for adhesion with hyphae, which were absent with fungi in their yeast morphology due to differences in the composition of the outermost mannoprotein layer [9]. This study confirms that the outermost mannoprotein layer on the fungal cell surface dictates whether or not favorable conditions exist for more intimate, specific interactions to develop [30], but more importantly identifies the importance of CbpD for the development of strong adhesion.

Chitin binding proteins or proteins containing chitin binding domains have been found in many bacterial strains and species. Species of the genera *Serratia*, *Lactococcus*, and *Streptomyces* have been reported to produce non-hydrolytic CbpD that act in synergy with chitinases [33-35]. Folders *et al.* [10] first identified the ability of *P. aeruginosa* to produce CbpD as well as a chitinase [12] and it was recently shown that CbpD played a role in bacterial colonization in the cystic fibrosis lung [11, 13, 36, 37]. All initial adhesion forces measured between the *Pseudomonas* strains and the *C. albicans* hyphal surface were low, and strong forces required time to develop. Although this type of bond-maturation is common also in the adhesion of inert particles and may involve purely physico-chemical processes like removal of interfacial water, unfolding of cell surface structures and conformational changes of binding protein and re-arrangement of entire cells or bacteria, it also points to the fact that chitin is hidden underneath the mannoprotein and β 1-3 glucan layers. This is in line with the general concept

that chitin is especially prominent in the inner side of the cell wall of *C. albicans*. Evidently, penetration of CbpD through the mannoprotein and β 1-3 glucan layer requires time, explaining in part the characteristic bond-maturation times observed. This is concurrent with observations on *Streptomyces* strains able to adhere to chitin-containing fungi hyphae (i.e. *Aspergillus proliferans*, *Neurospora crassa*), where chitin binding protein has also been described to invade deeper layer of fungal cell walls [35, 38]. The hidden position of chitin underneath the mannoprotein layer and the development of strong interactions underneath the mannoprotein layer explain why the interaction between CbpD and chitin is not reflected in the surface thermodynamic analysis. Surface thermodynamics is based on contact angle measurements, representing one of the most surface-sensitive techniques available and probing only the molecular properties of the outermost surfaces of synthetic and biological materials [39, 40].

It is difficult to assess whether the differences in adhesion forces observed here should be considered high or low, although clearly of influence on the interaction of *P. aeruginosa* with fungal cell surfaces. Since the introduction of the AFM and the development of techniques to prepare bacterial probes for interaction with surfaces, bacterial (co-)aggregation has been demonstrated to be sensitive to even minor differences in adhesion forces between strains. Co-aggregating and non-co-aggregating oral bacterial pairs had adhesion forces of around 1 and 4 nN, respectively [41]. Aggregation or its absence between *Enterococcus faecalis* strains, mediated by the aggregation substance Agg, a plasmid encoded surface protein, was accompanied by adhesion forces between the bacteria of around 2.5 nN versus 1.3 nN in strains lacking Agg [42]. A reduction in adhesion force between *Escherichia coli* and a silicon nitride AFM tip upon adsorption of cranberry juice components from higher than 0.5 nN to smaller than 0.5 nN has even been employed to explain beneficial effects of

cranberry juice on adhering urogenital pathogens [43]. Thus it can be concluded that the differences observed in the present study, are high and able to cause major changes in physical interaction between *P. aeruginosa* and fungal cells.

Concluding, in this study we show that CbpD in *P. aeruginosa* is responsible for strong physical interactions with *C. albicans* hyphae. The development of the interaction between CbpD and the hyphal cell wall requires time due to the fact that CbpD has to invade the outermost mannoprotein layer on the hyphal cell surfaces. In order to do this, thermodynamic conditions at the outermost cell surfaces have to be favorable, as established through surface thermodynamic analyses.

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Chapter 4

Staphylococcus aureus adherence to *Candida albicans* hyphae is mediated by the hyphal adhesin Als3p

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ABSTRACT

The bacterial species *Staphylococcus aureus* and the opportunistic fungus *Candida albicans* are currently among the leading nosocomial pathogens, often co-infecting critically ill patients with high morbidity and mortality. Our previous investigations had demonstrated *S. aureus* preferential adherence to *C. albicans* hyphae during mixed biofilm growth. In this study, we aimed to characterize the mechanism behind this observed interaction. *C. albicans* mutant strains were screened to identify the specific receptor on *C. albicans* hyphae recognized by *S. aureus*. The findings from these experiments implicated the *C. albicans* adhesin agglutinin-like sequence 3 (Als3p) in playing a major role in the adherence process as demonstrated with an immune assay. This association was quantitatively established using atomic force microscopy where the adhesion force between two species was significantly reduced for a *C. albicans* mutant strain lacking *als3*. This was further confirmed by confocal laser scanning microscopy, where *S. aureus* overlayed with a purified recombinant Als3 N-terminal domain fragment (rAls3p) exhibited strong interaction. Importantly, a strain of *Saccharomyces cerevisiae* heterologously expressing Als3p was utilized to further confirm this adhesin as a receptor for *S. aureus*. Although the parental strain does not bind bacteria, expression of Als3p on the cell surface conferred upon the yeast the ability to strongly bind *S. aureus*. In order to elucidate the implications of these *in vitro* findings in a clinically relevant setting, an *ex vivo* murine model of co-infection was designed using murine tongue explants. Fluorescent microscopic images revealed extensive hyphal penetration of the epithelium typical of *C. albicans* mucosal infection. Interestingly, *S. aureus* were only seen within the epithelial tissue when associated with the invasive hyphae. This differed from tongues infected with *S. aureus* alone or in conjunction with the *als3* mutant strain of *C. albicans*, where bacterial presence was limited to the outer layers of the oral tissue. Collectively, the findings generated from this study identified a key role for *C. albicans* Als3p in mediating this clinically-relevant fungal-bacterial interaction.

INTRODUCTION

Despite their clinical relevance, polymicrobial infections, defined as those infections caused by more than one microbial species, remain largely understudied [1]. Although they carry serious clinical implications in terms of impact on therapeutic measures, fungal-bacterial mixed infections specifically are poorly understood [2]. The lack of in depth investigations into inter-microbial interactions at the molecular level has contributed to our lack of understanding of these complex phenomena.

C. albicans is the most pathogenic human fungal species commonly colonizing host mucosal surfaces [3]. However, under conditions of immune disruption, *C. albicans* can rapidly transition into a pathogen causing an array of mucosal and disseminated infections with high mortality [4]. As a polymorphic species, *C. albicans* is capable of switching morphology between yeast and hyphal forms, a transition crucial to its pathogenesis. While the yeast form is most commonly associated with bloodstream and systemic disease, the hyphae are more adept at adhering to and penetrating host tissue and are therefore responsible for mucosal infections, most commonly oral candidiasis [5]. Similar to *C. albicans*, the bacterial species *Staphylococcus aureus* possesses a repertoire of virulence attributes which has contributed to its recent re-emergence as a significant pathogen particularly in immunocompromised individuals [6].

The clinical significance of intimate association between microbial species in general has remained underappreciated in most diseases; this is particularly significant when involving ubiquitous microbial species with high pathogenic potential such as *C. albicans* and *S. aureus.* In fact, the co-isolation of these diverse organisms from an array of acute and chronic diseases such as burn wounds, ventilator-associated pneumonia and bloodstream infections is well-documented [7-9]. The majority of these clinical conditions are considered to be biofilm-associated where adherence and colonization of the microorganism to a surface is a pre-requisite for the development of the infectious process [10]. Similarly, such infections that are polymicrobial in nature involve the

adherence of various microbial species to each other, where some level of interaction or cell-cell communication is expected to occur [1]. Therefore, elucidating the mechanisms of co-adherence and identification of specific receptors involved in inter-species interactions would aid in the design of novel strategies for impeding such processes.

Given their importance and prevalence as human pathogens and their abilities to co-adhere to surfaces, our initial investigations focused on characterizing the interaction between *S. aureus* and *C. albicans* at the molecular level. Specifically, global proteomic analyses were performed to identify the differential expression of proteins associated with their interaction during *in vitro* biofilm formation. The findings from these studies identified a significant number of proteins to be differentially-expressed by these two pathogens indicating the existence of a complex dynamic interactive process [11]. Furthermore, a distinct physical association was identified between these organisms, with *S. aureus* adhering to the hyphal filaments of *C. albicans*. However, a defined mechanism behind these interactions had yet to be characterized. To that end, this current study aimed to identify the specific *C. albicans* surface receptor for *S. aureus*. Since microscopic images had revealed a preferential association for *S. aureus* to *C. albicans* hyphae, our efforts focused on Als3p, one of the hyphal-specific adhesins of *C. albicans*. *ALS3* is a member of the *C. albicans ALS* (agglutinin-like sequence) gene family that encodes eight cell-surface glycoproteins with a N-terminal domain of adhesive function [12]. The Als proteins diffusely cover the surface of the fungal cell, and therefore are involved in adherence to host and abiotic surfaces. In fact, the role of these adhesins in initiating binding had been confirmed *via* their heterologous expression in *Saccharomyces cerevisiae* [13]. Importantly, these surface glycoproteins were also implicated in the adherence of *C. albicans* to bacterial species such as *Streptococcus gordonii* [14-16]**.** Therefore, based on these previous findings the aim of this current work was to validate the hypothesis that Als3p plays a key role in mediating the adherence of *S. aureus* to *C. albicans* hyphae.

MATERIALS AND METHODS

Strains, growth conditions and harvesting

Strains used in this study are listed in Table 1.

All media used were purchased from Difco (Sparks, MD, USA). Strains were stored as frozen glycerol stocks at -80°C and maintained on yeast peptone dextrose (YPD) plates. Cultures were grown in YPD broth overnight at 30°C. Cells were washed with sterile phosphate buffered saline (PBS:137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and grown in RPMI 1640 (without phenol red) medium supplemented with glutamine (0.3 mg/mL) and buffered with HEPES (25 mM, pH 7.4) (Invitrogen, Grand Island, NY) or yeast nitrogen base acids (YNB; Difco, Sparks, USA) pH 7, containing 0.5% D-glucose at 37ºC to induce hyphal formation. Methicillin-resistant *S. aureus* strain M2, originally isolated from a patient with osteomyelitis at University of Texas Medical Branch was used in all studies [27]. The strain was stored as a glycerol stock at -80°C and maintained on trypticase soy agar (TSA) containing 5% sheep's blood. Cultures were grown overnight at 37°C in trypticase soy broth (TSB), diluted 1:100 in fresh media and grown to mid-log phase prior to use.

Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM)

Microscopic analysis was performed in order to visualize the interactions between *S. aureus* and *C. albicans* as they co-adhered to a surface. Log-phase *C. albicans* and *S. aureus* cells were washed in PBS and diluted to an OD_{540} of 0.1 and OD_{600} of 0.12, respectively. Fifty µL from each culture was inoculated into the wells of a 6-well polystyrene plate containing a catheter disc and 5 mL RPMI medium for SEM or into wells of a LabTek II 8-well chamber slide (Nunc, Rochester, NY) containing 200 µl RPMI for CLSM.

Table 1 Yeast strains used in this study.

Strain	Designation	Genotype	Parent	Reference
***C. albicans* strains**				
SC5314	Wild-type	*URA3/URA3 ARG4/ARG4 HIS1/HIS1*	-	[17]
CAI4	CAI4	*ura3/ura3*	SC5314	[18]
DAY185	Parent	*URA3/ura3 ARG4/arg4 HIS1/his1*	SC5314	[19]
1467	*Δals1*	*ura3/ura3 als1/als1-URA3*	CAI4	[20]
2757	*Δals2*	*ura3/ura3 als2/als2-URA3*	CAI4	[21]
1843	*Δals3*	*ura3/ura3 als3/als3-URA3*	CAI4	[20]
2034	*Δals4*	*ura3/ura3 als4/als4-URA3*	CAI4	[22]
2373	*Δals5*	*ura3/ura3 als5/als5-URA3*	CAI4	[23]
1420	*Δals6*	*ura3/ura3 als6/als6-URA3*	CAI4	[23]
1429	*Δals7*	*ura3/ura3 als7/al73-URA3*	CAI4	[23]
2028	*Δals9*	*ura3/ura3 als9/als9-URA3*	CAI4	[24]
CAH7-1A	*Δhwp1*	*ura3/ura3 hwp1/hwp1-URA3*	CAI4	[25]
***S. cerevisiae* strains**				
UB2155 WT	Wild-type	UB2155 + pBC542 (empty vector)	BY4742	[26]
UB2156 2156	2156	UB2155 + pBC542-ALS3sm	UB2155	[26]
UB2157 2157	2156	UB2155 + pBC542-ALS3lg	UB2155	[26]

Polyvinyl chloride (PVC) catheter discs (BenTec Medical Inc., Wakefield, MA) were sterilized by soaking in 100% ethanol for 10 min, rinsed in sterile water and allowed to dry overnight. Plates were shaken at 120 rpm for 5 min then incubated at 37°C for 16 h with shaking at 50 rpm. Following incubation, media was removed and wells were gently washed with PBS to remove non-adherent cells. Biofilms were then fixed in 4% paraformaldehyde for 1 h then extensively rinsed in PBS and processed for SEM. Biofilms

on the cather discs were fixed and immediately processed for SEM by cryofreezing and sputter coating. Biofilms were analyzed and imaged using SEM (Quanta 200; FEI, Hillsboro, OR, USA) at 4000X magnification.

For CLSM, biofilms were fixed in 4% paraformaldehyde and stained with a cocktail of fluorescent probes: calcofluor white (stains chitin in fungal cell wall), Syto9 (DNA stain) and concanavalin A conjugated to Texas Red (stains carbohydrate moieties including microbial matrix). Discs were scanned and imaged using a Zeiss 510 Meta (Carl Zeiss, Thornwood, NY) using a combination of differential interference contrast and/or DAPI/FITC/Texas Red filter sets. In order to assess the effect of serum on the interaction between the two species, experiments were also performed as described above in 8-well chamber slides in RPMI with the addition of bovine serum (25%) to the growth medium.

Atomic force microscopy (AFM)

AFM was performed in order to measure force curves between *S. aureus* and selected *C. albicans* strains [the mutant starin1843 (Δ*als3*) and the parent strain DAY185] adhered on glass slides, as described previously with some modifications [28]. Briefly, adhesion forces were measured in PBS (pH 7) at room temperature with z-scan rates of less than 1 Hz using an optical lever microscope (Nanoscope IV, Digital Instruments, Woodbury, NY, USA) after 0, 5, and 60 s surface delay times on the same, randomly chosen spot on a hypha. To ensure that no bacteria detached from the cantilever during the experiment, ten control force-distance curves were made with 0 s contact time (immediate retraction of cantilever tip) after each set of measurements. Prior to using a bacterial probe for force measurement, it was used to image hyphae in order to verify that the probe established single cell contact with the fungal cell. Double contour lines observed in imaging with a bacterial probe indicate double cell contact and probes exhibiting such behaviour were discarded. For each experiment, three bacterial probes were used and

median adhesion forces were calculated from at least 80 force-distance curves. Calibration of cantilevers was done using the thermal tuning method (Nanoscope V6.13r1), utilizing spring constants ranging from 0.04 to 0.06 (N/m).

Production of recombinant Als3 N-terminal domain fragment (rAls3)

To determine whether Als3p is required for *S. aureus* adherence to *C. albicans*, a recombinant protein was produced in *Pichia pastoris* and used in some experiments. Detailed information on the production of the protein is provided as supplementary material. Following expression, the protein of interest was purified by column chromatography and its identify was confirmed as Als3p by mass spectrometry. The N-terminal domain of Als1p (amino acids 18-329 of the protein) was produced in *P. pastoris* as described previously and used as a control [29].

Adherence enzyme linked immunosorbent assay (ELISA)

An ELISA-based assay was developed in order to confirm inter-species adherence and the involvement of Als3p in the process. The experiments were performed using the Mouse Immunoglobulin Isotyping ELISA kit (BD Pharmingen, San Diego, CA, USA) according to manufacturer protocol with some modifications. *C. albicans* cells were grown as described above, washed in sterile PBS, and diluted to an OD_{540} of 0.1 in fresh RPMI. One hundred µl aliquots were placed in individual wells of a 96-well high-protein-binding ELISA plates and incubated overnight at 37°C to allow cells to adhere and coat the surface of the wells. *S. aureus* cells were grown and harvested as described above then homogenized using a PT1200 Polytron homogenizer (Kinematica Inc., Bohemia, NY, USA) and diluted to an OD_{600} of 0.12 (approx. $1x10^8$ CFU/mL) in sterile PBS. Wells with adhering *C. albicans* cells were extensively washed in wash buffer (1X PBS, 0.05% Tween-20, 1% BSA) to remove non-adherent cells. Wells were then blocked with 1% bovine serum albumin (BSA) for 1 h. *S. aureus* cell suspensions (100 µl) were added to

each well and plates were incubated for 1 h at room temperature with gentle agitation (50 RPM). Following incubation, wells were extensively washed with wash buffer to remove non-adherent *S. aureus* and incubated with a 1:1000 dilution of a horseradish peroxidase (HRP)-conjugated mouse anti-*S. aureus* IgG polyclonal antibody (Pierce Biotechnology, Rockford, IL, USA). 3,3',5,5'tetramethylbenzidine reagent was added to each well and plates were incubated for 5 min to allow for color development indicating *S. aureus* binding to the hyphae-coated wells. A stop solution (2N H_2SO_4) was added and absorbances were measured on a Biotek ELx808 plate reader at 550 nm and analyzed with the Gen5 software suite.

In order to demonstrate that *S. aureus* adherence to the hyphae was mediated by Als3p, ELISAs were also performed by coating wells with various concentrations of purified rAls3p, rAls1p, or a synthesized control peptide (NH_2-MRFPSIFTAVLFAASSALAAPVNTTTEDAEQKILSEEDLNSAVDHHHHHH-OH) diluted in protein storage buffer (NeoBioscience, Cambridge, MA, USA). A peptide including the N-terminal and C-terminal sequences of the recombinant protein but lacking Als3p sequences was used as the control. Plates were processed as described above.

Indirect immunofluorescence of rAls3p

In addition to ELISA, the generated rAls3p was used for indirect immunofluorescence. In these experiments, *S. aureus* cells grown on the surface of Permanox-coated chamber slides (Thermo Scientific, Rochester, NY, USA) were extensively washed with PBS to remove non-adherent cells and overlayed with 50 µg of purified rAls3p or control peptide. Following incubation for 1 h at room temperature, slides were rinsed 3 times in PBS, treated with the cell-permeable DNA stain Syto9 (Invitrogen, Carlsbad, CA) and fixed in 4% formalin. A primary mouse IgG antibody specific for the c-myc epitope tag on rAls3p (Biolegend, San Diego, CA, USA) was added and incubated for 4 h at 4°C. Slides were then washed three times in wash buffer followed by incubation with a Cy3-

conjugated secondary goat anti-mouse IgG antibody (Biolegend) for 1 h at room temperature then imaged with a Zeiss 510 Meta confocal microscope using differential interference contrast and Cy2/Cy3 line switch filters.

Interactions of *S. cerevisiae* with *S. aureus*

To confirm the identification of Als3p as a receptor for *S. aureus* on *C. albicans* hyphae, strains of the yeast *S. cerevisiae* heterologously expressing the small (*ALS3sm*) and large (*ALS3lg)* alleles of the protein on their cell surface were utilized [26]**.** Yeast cells grown for 16h were washed in PBS, transferred to fresh media and incubated with shaking (50 RPM) for 3 h at 30°C. 1 ml of 5 x10^8 cells/ml *S. aureus* were added to *S. cerevisiae* suspensions and cells incubated together for 1 h. *S. cerevisiae* cells were then washed to remove unattached bacteria, resuspended in 1/10 original volume and visualized by light microscopy.

***Ex vivo* model of co-Infection**

In order to determine whether the interaction between *S. aureus* and *C. albicans* observed on abiotic surfaces can occur on host tissue, an *ex vivo* murine tongue infection model was designed. All animal procedures were approved by the University of Maryland--Baltimore Animal Care and Use Committee. Eight-week old female CD-1 (ICR) mice purchased from Charles River Laboratories were housed in groups of 5 and provided ad libitum feed and water containing 0.3 mg/mL ampicillin (Teknova, Hollister, CA, USA). Mice were euthanized and tongues excised and placed in wells of a 12-well polystyrene plate with 2 mL of RPMI medium. Cell suspensions of *C. albicans* strains were standardized and 50 µl aliquots of either or in combination were added to the wells with tongues and incubated for 1 h at 37°C with gentle agitation. Tongues were then washed several times in sterile PBS and transferred to fresh RPMI and incubated overnight at 37°C with 5% CO_2. Following incubation, tongues were fixed in 4% formalin,

embedded in paraffin and sectioned. Tissue sections were deparaffinized with xylene and stained with hematoxylin and eosin for histopathological analysis using light microscopy. Alternatively, sections were also hybridized with species specific peptide nucleic acid probes for fluorescent *in situ* hybridization (PNA-FISH) according to the manufacturer protocol (AdvanDx, Woburn, MA, USA) and examined by CLSM as described earlier. The *S. aureus* specific probe was Cy2-labeled and *C. albicans* probe labeled with Cy3.

Statistics

Experiments were performed on three separate occasions in triplicate unless noted otherwise. A one-way ANOVA and Dunnett's post-test was used to compare ELISA data. Adhesion forces (>80 measurements per hyphal cell) measured by AFM were not normally distributed according to the Shapiro-Wilk test and hence results are presented as median and interquartile ranges [28]. The results were compared using a non-parametric Mann-Whitney test. To compare quantitative counts using *S. cerevisiae* Als3p expressing strains, a one-way ANOVA and Bonferroni's post-test was used. For all experiments, a p value of < 0.05 was considered statistically significant. Statistics were calculated using GraphPad Prism 5.0. Images were processed using Adobe Photoshop CS5.

RESULTS

The physical interaction between *S. aureus* and *C. albicans* was visualized through high-resolution SEM revealing a three-dimensionally distributed pattern of *S. aureus* hyphal attachment. As can be seen in Figure 1a, *S. aureus* cells bordered the basal layer of the hyphae-substratum interface as well as adhered to the upper portion of the hyphal surface. Similarly, CLSM images (Figure 1b) not only confirmed the adherence but also identified the involvement of the hyphal Als3p adhesin, as a substantial decrease in the

level of adherence was observed for *S. aureus* to the *C. albicans* strain lacking the Als3 protein (Figure 2c) compared to the parental strain (Figure 2j). In addition to the *als3* mutant, strains lacking other members of the *ALS* gene family were also examined for their ability to adhere to *S. aureus*. In contrast to the minimal adherence pattern seen with *als3*, *S. aureus* adherence to these other strains was comparable to that with the parental strain (Figure 2a,b,d-i). Images with *C. albicans* alone (Figure 2k) or *S. aureus* alone Figure 2l) are included as controls.

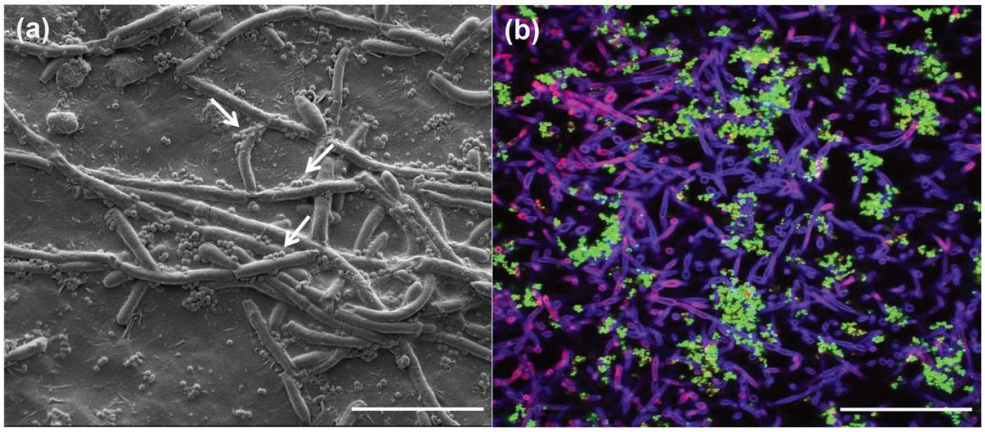

Figure 1 Microscopic analysis of the adherence architecture between *S. aureus* and *C. albicans*. **(a)** Scanning electron microscopic image of catheter discs demonstrating *S. aureus* M2 preferential attachment to *C. albicans* DAY185 hyphae (white arrows). **(b)** Confocal laser scanning image of *S. aureus* M2 and *C. albicans* DAY185 adhering to the surface of chamber slides stained with a cocktail of calcofluor white, Syto9, ConA-Texas Red stains. *S. aureus* M2 (green) can be seen attached to *C. albicans* DAY185 hyphae (purple). Scale bar represents 20 µm.

In addition to visual microscopy, the adhesion forces between *S. aureus* and the *C. albicans* strains were measured using AFM. Typical force-distance curves of *S. aureus* with *C. albicans* are shown in Figure 3a. Adhesion forces between *S. aureus* and hyphae of *C. albicans* Δ*als3* increased from (-0.1 nN) at 0 s contact time (initial contact) to (-3.2 nN) after 60 s surface delay (Figure 3b). Irrespective of delay times, these forces were significantly smaller than those between *S. aureus* and *C. albicans* parental strain where

a (-1.3 nN) force was generated at initial contact (0 s) and increasing to (-7.2 nN) after 60 s surface delay time (Figure 3a,b). Adhesion forces between *S. aureus* and the Δ*als1* and Δ*hwp1* mutants were similar to the *C. albicans* parental strain (data not shown).

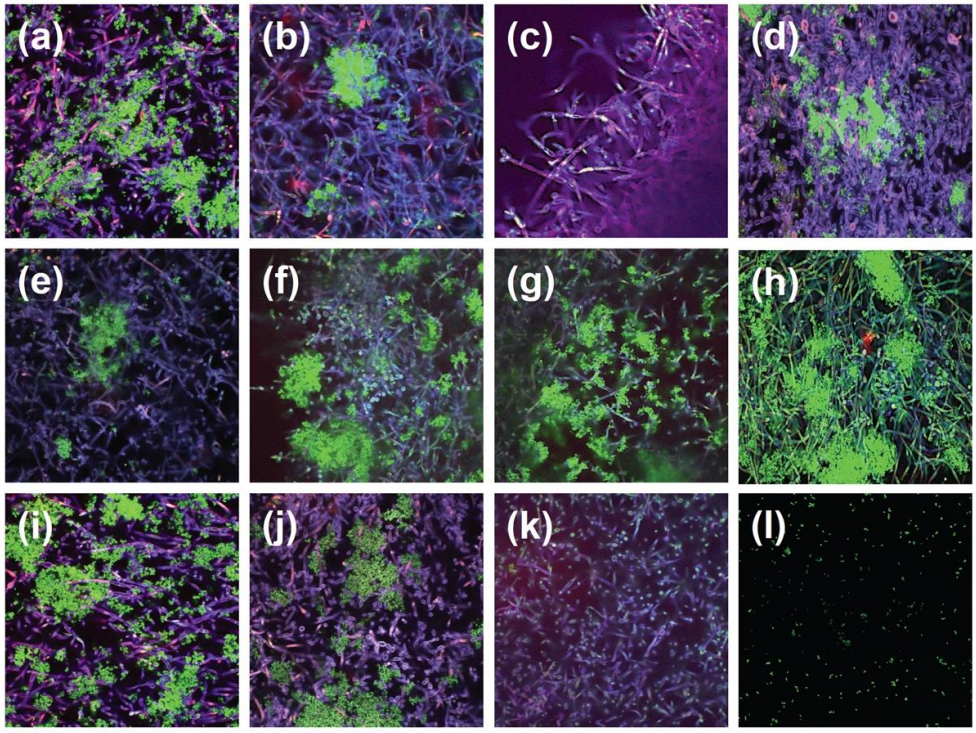

Figure 2 Comparative analysis of *S. aureus* adherence to *C. albicans* strains as assessed by confocal microscopy. Imaging of *S. aureus* M2 adherence to *C. albicans* biofilms demonstrating decreased binding to the Δ*als3* strain **(c)** as compared to the parent strain (DAY185) **(j).** In contrast, a comparable pattern of adherence to the *C. albicans* parent strain was seen for *S. aureus* with the remaining mutant stains: Δ*als1* **(a)**, Δ*als2* **(b)**, Δ*als4* **(d)**, Δ*als5* **(e),** Δ*als6* **(f),** Δ*als7* **(g),** Δ*als9* **(h),** Δ*hwp1* **(i).** *C. albicans* parent strain (DAY185) **(k)** and *S. aureus* **(l)** grown alone on chamber slides were included as controls.

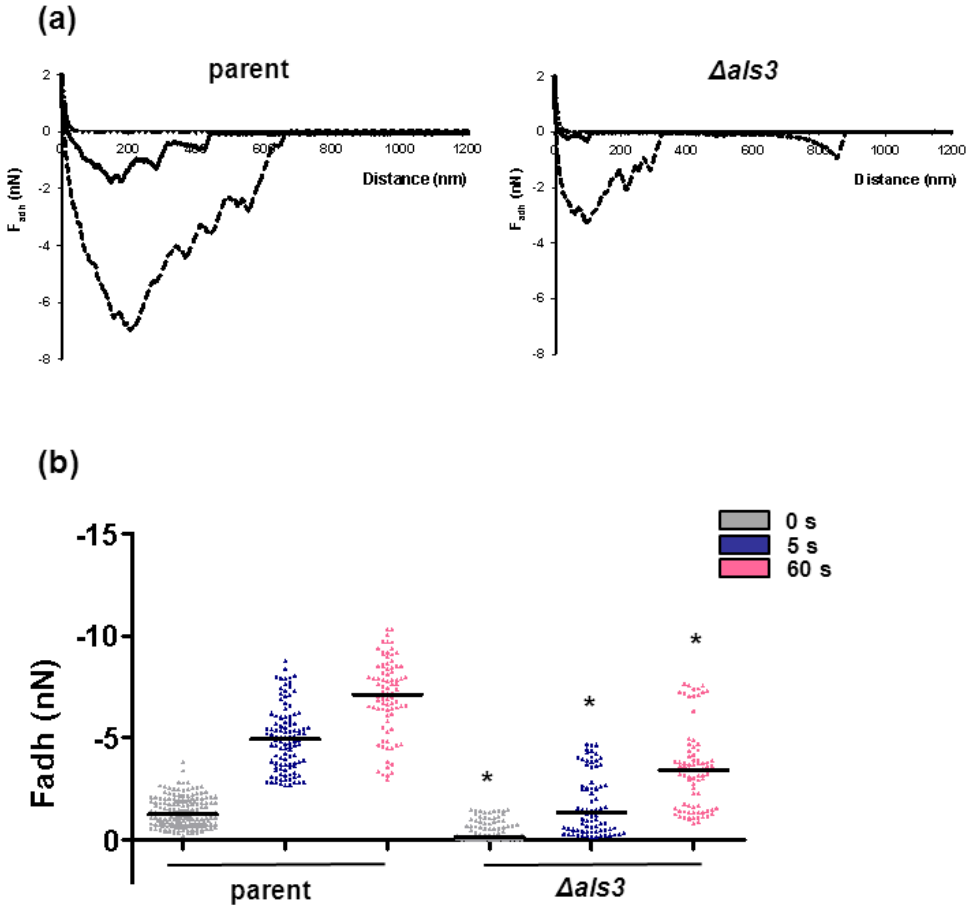

Figure 3 Atomic force measurements of *C. albicans* and *S. aureus* interaction. **(a)** Typical examples of force-distance curves between *S. aureus* M2 and *C. albicans* DAY185 strains recorded after 0 s (solid line) and 60 s (dashed line) surface delay time. **(b)** *C. albicans* mutant lacking Als3p exhibited significant decrease in adhesion force (F_{adh}) with *S. aureus* M2 at all time points tested compared to the parent (DAY185). Cantilever tips with *S. aureus* were allowed to interact with a randomly selected hypha and the adhesion forces measured ten times; results represent the median of at least 80 individual force-distance curves from three experiments with independently grown cultures. Error bars represent the interquartile range (*, $p < 0.05$).

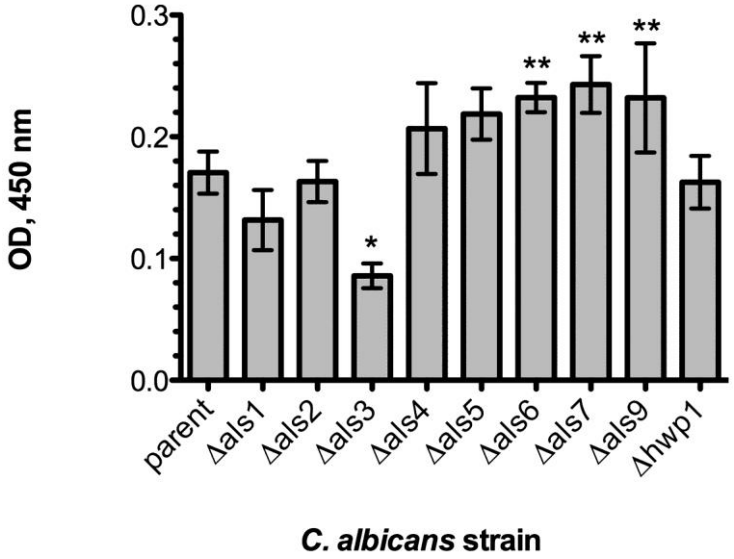

Figure 4 ELISA assessment of staphylococcal adherence to *C. albicans* DAY185 parent and mutant strains. A statistically significant reduction in the level of bound *S. aureus* M2 (*, $p < 0.05$) to the strain lacking Als3p (Δ*als3*) is shown as compared to the parent; some strains exhibit increases in *S. aureus* M2 binding (**, $p < 0.05$). Groups were compared using a one-way ANOVA and Dunnett's post-test. Error bars represent standard error of the mean.

Further semi-quantitative analysis for hyphal binding between *S. aureus* and *C. albicans* parental and mutant strains was performed using an immunoassay. Comparisons of the absorbance readings obtained from assays following color development, the intensity of which reflects the extent of adherence, demonstrated significant differences in the level of *S. aureus* adherence to *Δals3* compared to the parental strain (Figure 4); adherence to other *C. albicans* mutant strains was comparable, or in some cases even increased, relative to the parental strain. In addition to using whole *C. albicans* cells, assays were also performed where various concentrations of the rAls3 protein and its control peptide (lacking the Als3p sequence) were used to coat the wells of ELISA plates. These results demonstrated a rAls3p dose-dependent level of adherence for *S. aureus* to the coated wells (r^2=0.836), while staphylococcal binding to the wells coated with the control peptide was nearly absent at

all concentrations tested (Figure 5a). Since the structure of Als1p closely resembles that of Als3p, purified rAls1p was also used as a comparative control. Although some adherence was noted with that peptide, the level was up to 3 fold less than that for Als3p, requiring peptide concentrations above 2.5 µg/ml to support appreciable staphylococcal binding (Figure 5a).

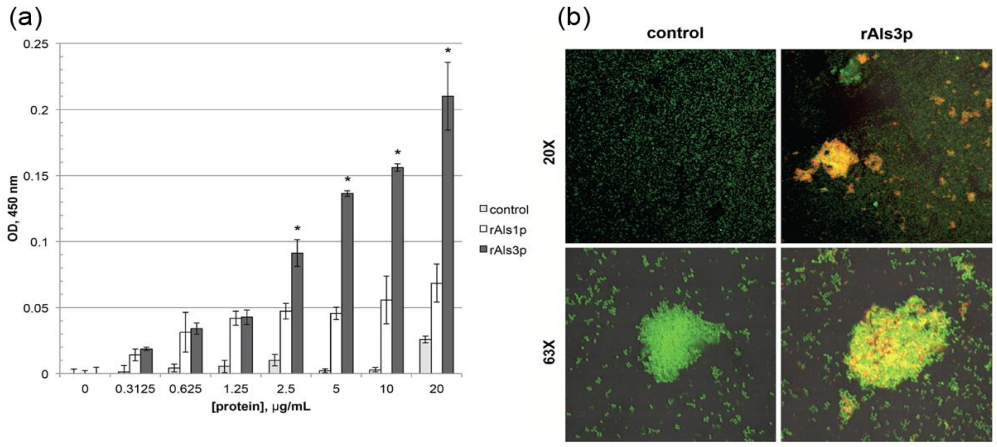

Figure 5 Evaluation of *S. aureus* adherence to the purified recombinant protein (rAls3p) by ELISA and indirect immunofluorescence. **(a)** *S. aureus* bound to rAls3p (dark gray bars) in a dose-dependent fashion but not to wells coated with a control peptide lacking the N-terminal Als3p sequence (light gray bars). Although *S. aureus* bound to rAls1p (white bars), the level of adherence was non-dose dependent and significantly less than binding to rAls3p (*, $p < 0.05$). **(b)** indirect immunofluorescence images of *S. aureus* cells interacting with rAls3p or control peptide stained with the DNA stain Syto9 (green). Following probing with a primary mouse IgG antibody specific for the c-myc epitope tag and a Cy3-conjugated secondary antibody (red), cells demonstrating peptide binding appear yellow due to the green-red fluorescence merge.

An antibody specific for the c-myc epitope on the C-terminus of rAls3p was used to image the adherence of this peptide to *S. aureus* cells. The control peptide was also included in these assays. Upon staining with Syto9, all *S. aureus* cells appeared green (Figure 5b). However, only *S. aureus* cells bound to rAls3p fluoresced red upon incubation with the Als3p-specific antibody and a Cy3-labeled secondary antibody. This

was confirmed by images demonstrating that only the addition of rAls3p to the cells resulted in red fluorescence (Figure 5b) with no red observed in the absence of rAls3p.

To further confirm the *C. albicans* adhesin Als3p as a mediator of co-adherence with *S. aureus*, the interaction of a *S. cerevisiae* heterologous host strain expressing *C. albicans* Als3p incubated with *S. aureus* was investigated. Microscopic images demonstrated that *S. aureus* did not bind parental *S. cerevisiae*. However, *S. aureus* bound strongly to cells expressing both the large and small alleles of Als3p (Figure 6a,b).

Tongues excised from euthanized mice were infected *ex vivo* with either species alone or co-infected with both in order to assess the potential implications of this interaction on a host. As expected, examination of PNA-FISH stained tissue sections demonstrated efficient adherence of *S. aureus* (green) to the outer layers of the tongue epithelium with no bacteria seen in the sub-epithelial tissue (Figure 7a). These images were consistent with those observed with hematoxylin and eosin staining where lack of inflammatory infiltrates confirmed a non-invasive presence of *S. aureus* on the tissue (Figure 7a). Similar to images from experiments using abiotic surfaces, sections from co-infected tongues showed *S. aureus* adhering to the hyphae (Figure 7b). Interestingly, however, *S. aureus* also could be seen in the sub-epithelium in association with the invading hyphae (Figure 7b, arrow). In contrast, although the hyphae of the *Δals3* strain penetrated the epithelial tissue similar to parental strain, minimal *S. aureus* cells were seen attached to the hyphae. Importantly, no staphylococci presence was detected beneath the outer layers of the tissue (Figure 7c).

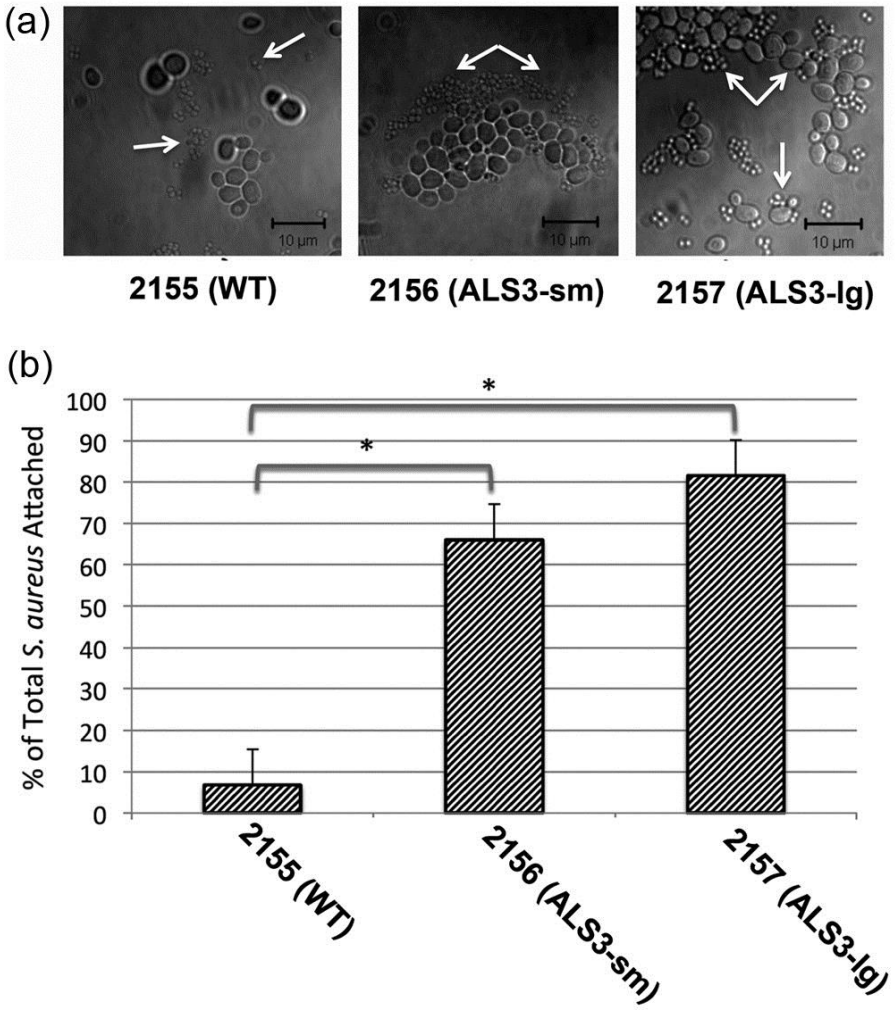

Figure 6. Interactions of *S. aureus* with *C. albicans* Als3p adhesin expressed on the surface of surrogate host *S. cerevisiae*. **(a)** *S. cerevisiae* cells expressing the candidal adhesin Als3p were incubated for 1 h with staphylococci. Cells were then harvested and visualized by light microscopy. Although *S. aureus* did not bind to the wild type *S. cerevisiae*, it bound strongly to cells expressing Als3p, both large and small alleles. White arrows show adherent *S. aureus*. **(b)** Percent of total bacteria bound to yeast cells, demonstrating a significant increase in the number of *S. aureus* adhering to Als3p-expressing yeast cells as compared to wild type (*, $p < 0.001$).

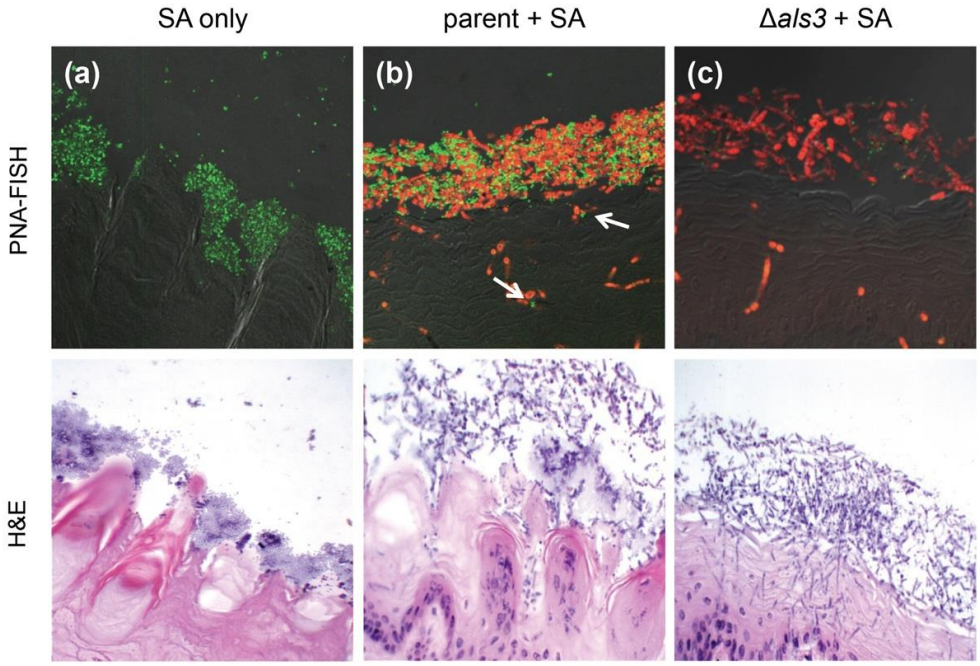

Figure 7. *Ex vivo* murine model of co-infection. Mouse tongues were infected *in vitro* with *S. aureus* and in combination with the *C. albicans* parent strain (DAY185) or the *als3* mutant strain. Sectioned tissue was stained with species-specific fluorescent (PNA-FISH) or histopathology (hematoxylin and eosin) stains and examined by microscopy. **(a)** Infection with *S. aureus* (green) was confined to the outer layers of the epithelial surface with no evidence of tissue invasion as confirmed by a lack of inflammatory infiltrate. **(b)** *S. aureus* (green) with parent strain (parent+SA) is seen attached to the hyphae (red) and in some instances detected beneath the epithelium associated with penetrating hyphae (white arrow). **(c)** However, although the *als3* mutant (Δ*als3* + SA) hyphae penetrated the tissue, relatively few staphylococci are seen attached to the hyphae with no bacterial presence noted beneath the epithelial layers.

DISCUSSION

Candida spp. and *Staphylococcus spp*. are currently among the leading pathogens causing nosocomial and bloodstream infections [30]. Their co-isolation from patients during various disease states is increasingly reported within the context of polymicrobial infections [8, 31-33]. Building on our earlier investigations demonstrating a robust attachment of *S. aureus* cells to *C. albicans* hyphae during polymicrobial biofilm formation, this current study was designed to characterize this inter-species interaction and identify the hyphal receptor for *S. aureus* [11]. To that end, extensive analyses were performed using several microscopic approaches, immunoassays, protein expression techniques, and a tissue infection model.

Images from confocal and electron microscopy clearly demonstrated the close association between these two diverse species (Figure 1). This association was previously reported by Harriott and Noverr, where *S. aureus* was shown to be embedded in the fungal biofilm matrix [34]. Significantly, this close association between *C. albicans* and *S. aureus* in a biofilm conferred *S. aureus* with resistance to antibiotics. These findings carry significant clinical implications in terms of therapeutic measures and therefore, warranted further investigations to elucidate the mechanism behind this association. Importantly, the identification of a specific adherence receptor would aid in the design of novel strategies for impeding co-adherence and subsequent infection.

The *C. albicans* Als proteins and the hyphal wall protein (Hwp1) are the main cell wall proteins implicated in the adherence of *C. albicans* to host tissue and several bacterial species, namely the oral streptococci [26, 35]. The streptococcal surface molecules largely involved in this interaction were demonstrated to be surface protein A and B (SspA/SspB) [14]. These types of interactions are considered synergistic as streptococci were shown to not only enhance *C. albicans* colonization of the oral cavity but also promote hyphal growth by excreting lactate as a carbon source [14, 15]. In addition to Gram-positive bacteria, association of *C. albicans* with Gram-negative

species has also been reported, most prominently with the pathogen *Pseudomonas aeruginosa* [36]. However, in contrast to the seemingly synergistic interaction with *S. aureus*, that with *P. aeruginosa* was described to not only be antagonistic in nature with the bacteria capable of killing the hyphae, but to also involve quorum sensing molecules and virulence factors [37, 38].

Based on these earlier studies, we tested several *C. albicans* cell wall mutants in an attempt to identify a specific receptor for *S. aureus* on the hyphae. Our initial screen demonstrated that the strain lacking Als3p had a reduced ability to adhere to *S. aureus*, attributing a crucial role for that protein in the adherence process (Fig. 4); these results were further confirmed by microscopy (Figure 2). In contrast, the other mutants of Als proteins as well as the strain lacking the Hwp1p exhibited adherence levels comparable to that for the parent strain. It is important to note however, that some bacterial cells were seen attached to the hyphae of the *als3* mutant indicating that additional molecules (including biofilm matrix) may also be involved in the process (Figure 7c). Alternatively, these observations could also be due to non-specific attachment due to hydrophobic or electrostatic interactions.

Although microscopic images clearly revealed differences in adherence potential between the strains, AFM was utilized in order to obtain a quantitative measure. AFM is a powerful tool that has proven useful in determining intraspecies adhesion forces [39-41]. Based on these measurements, only the deletion of *als3* demonstrated a significant reduction in the staphylococcal-hyphal binding force (Figure 3). Further confirmation for the involvement of Als3p came from ELISA testing where *C. albicans* cells as well as a recombinantly-expressed peptide of the N-terminal domain of Als3p containing the receptor-binding function, were used for capturing overlayed *S. aureus* cells. These results demonstrated reduced binding of *S. aureus* to the *als3* mutant of *C. albicans* as compared to the parent strain (Figure 4) and a dose-dependent adherence of *S. aureus* to rAls3p-coated wells (Figure 5). Since Als1p is closely related to Als3p, its ability to

directly bind *S. aureus* was also tested. Although the *als1* mutant strain did not exhibit reduced capacity to bind to *S. aureus* as demonstrated by ELISA measurements (Figure 4) and microscopy (Figure 2a), the purified protein, when coated on the wells of microtiter plates, did exhibit some capacity to support staphylococcal binding (Figure 5a). However, these observations could be due to masking of Als1's contribution to *S. aureus* binding by functional Als3 protein at the fungal surface or conformational differences between the native protein and purified peptide. Collectively, these findings indicated that Als3p is involved in the interaction between *C. albicans* with *S. aureus*. Importantly, this hypothesis was firmly validated using a *S. cerevisiae* strain heterologously expressing the *C. albicans* Als3p where *S. aureus* was shown to strongly bind only to the surface of the Als3p-expressing yeast cells (Figure 6).

Since it is known that both *C. albicans* and *S. aureus* have receptors to various proteins in serum, we sought to determine whether the observed attachment could be enhanced by cross-bridging independent of adhesin expression [42, 43]. Incorporation of serum in our polymicrobial experiments clearly demonstrated that in the absence of serum, *S. aureus* adhered only to the parental strain and not to the Δ*als3* mutant whereas in the presence of serum, *S. aureus* avidly colonized the hyphae of both *C. albicans* strains (Figure S1). This noted ability of serum to mediate non-specific co-adherence may explicate the discrepancies between our results and those from previous studies, where in the presence of serum, *S. aureus* associated equally-well when grown with the Δ*als3* mutant strain [21]. Interestingly, although Als3p has been implicated in *C. albicans* adherence to various bacterial species, the mechanism of adherence appears to be vastly different between Gram-negative and Gram-positive bacteria. A study by Brand *et al*. demonstrated that the interaction between *P. aeruginosa* and *C. albicans* is independent of Als3p as strains deficient in the production of the hyphal proteins Hwp1, Als3 and Hyr1 were as susceptible to killing by this bacterial pathogen as the parent

strain [44]. These observations indicate that *P. aeruginosa* binding to the hyphae is likely mediated by glycan moieties rather than specific surface proteins.

Perhaps the most significant findings with potential clinical implications come from the experiments examining the ability of *C. albicans* and *S. aureus* to co-adhere to host tissue (Figure 7). Microscopic images from sectioned mouse tongue tissue paralleled those previously seen *in vitro* where an extensive network of hyphae from the *C. albicans* parental strain was seen covering the tongue surface with *S. aureus* interspersed throughout the hyphal matrix (Figure 7b). Importantly, bacterial cells were at times seen beneath the epithelial barrier of the tissue where hyphae had penetrated (Figure 7b, arrow). In contrast, although the *als3* mutant did not exhibit defects in hyphal formation, minimal bacterial cells were seen adhering to the hyphae (Figure 7c). These findings are of considerable significance since although *C. albicans* hyphal filaments are considered to be highly invasive of mucosal tissue, *S. aureus* infections are strongly correlated with a prerequisite breach of innate biological barriers [45-47]. Therefore, it is conceivable to speculate that the association of staphylococcal cells with *C. albicans* hyphae, as they penetrate host tissue, may allow *S. aureus* to gain entry into deeper tissues and initiate infection with dire consequences to the host, particularly in critically-ill patients.

Summarizing , this study identifies the hyphal adhesin Als3p as a receptor for the bacterial pathogen *S. aureus* on *C. albicans* hyphae. The interaction between such diverse and important pathogens holds significant clinical implications and therefore, characterizing their complex interactions is the first step in understanding the nature of their co-existence in the host. The findings generated will not only advance our understanding of the molecular mechanisms underlying the strong interaction between these pathogens, but may lead to the development of novel strategies to impede microbial co-adherence. Future studies are now focused on identifying the receptors on *S. aureus*.

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SUPPLEMENTARY INFORMATION AND FIGURES

Production of recombinant Als3 N-terminal domain fragment (rAls3). To determine whether Als3p is required for *S. aureus* adherence to *C. albicans*, a recombinant protein was produced and used in some experiments. Primers ALS3EcoRIF (5'-GAGAGAATTCATGCTACAACAATATA-3') and ALS3XbaIR (5'-GAGATCTAGAGTAGTACTTGTCTGTAACT-3') were used to amplify a 1016 bp fragment of ALS3 from *C. albicans* genomic DNA. The fragment was digested with EcoRI–XbaI and ligated with similarly digested plasmid pPICZαA (Invitrogen). The resulting plasmid was linearized with SacI and transformed into *Pichia pastoris* strain GS115 using the lithium acetate polyethylene glycol method as described previously [48]. Zeocin-resistant colonies were screened by PCR for integration of the plasmid at the AOX1 locus, downstream of the methanol-inducible AOX1 promoter. The correct clone was inoculated into 25 ml of buffered minimal glycerol medium (BMGY) and incubated at 30 °C overnight with shaking at 225 r.p.m. Cells were harvested by centrifugation, resuspended in fresh BMGY, inoculated into 1-litre batches of BMGY and grown at 30 °C overnight with shaking at 225 rpm. Following incubation, cells were harvested, washed in sterile PBS and inoculated into buffered minimal methanol medium at an OD600 of 1.0 in 1-litre batches. Cells were grown at 30 °C for 24 h with shaking at 225 rpm, harvested then washed with PBS and used immediately or frozen at -80 °C until needed. Cells were resuspended in lysis buffer (8 M urea, 5 mM DTT, 150 mM NaCl, 50 mM Tris/HCl, pH 7.4) then broken using 0.5 mm zirconia beads (BioSpec Products) in a FastPrep FP120 bead mill (ThermoSavant). Cell lysate was centrifuged at 14000 g for 10 min to obtain a crude protein fraction. The rAls3 protein of interest was further purified using gravity-flow HisPur column chromatography and eluted from the column with elution buffer containing 300 mM imidazole according to the manufacturer's instructions (Pierce Biotechnology). Equal amounts of protein (20 μg) were separated by SDS-PAGE, transferred to PVDF membranes and subsequently probed by Western

blotting with a primary mouse IgG anti-c-myc antibody (BioLegend) and a secondary rabbit HRP-coupled IgG anti-mouse antibody (KPL). The presence of the recombinant protein was detected using the PicoWestern (Pierce Biotechnology) protocol and a Fluorchem 8900 CCD camera (AlphaInnotech). A band corresponding to approximately 39 kDa recognized by Western blotting of the lysate was excised, sequenced by MALDI-ToF/ToF analysis and confirmed as Als3p as described previously [11]. The elution fractions were pooled, concentrated by centrifugal 10 kDa molecular mass filters (Sigma), and the buffer exchanged with protein storage buffer (50 mM HEPES, 150 mM NaCl, 5 mM DTT, 10% glycerol, 0.1% NP-40, pH 7.4). The N-terminal domain of Als1p (amino acids 18–329 of the protein) was produced in *P. pastoris* as described previously and used as a control [29]. Proteins were quantified colorimetrically by the Advanced Protein Assay Reagent #2 (Cytoskeleton Inc.).

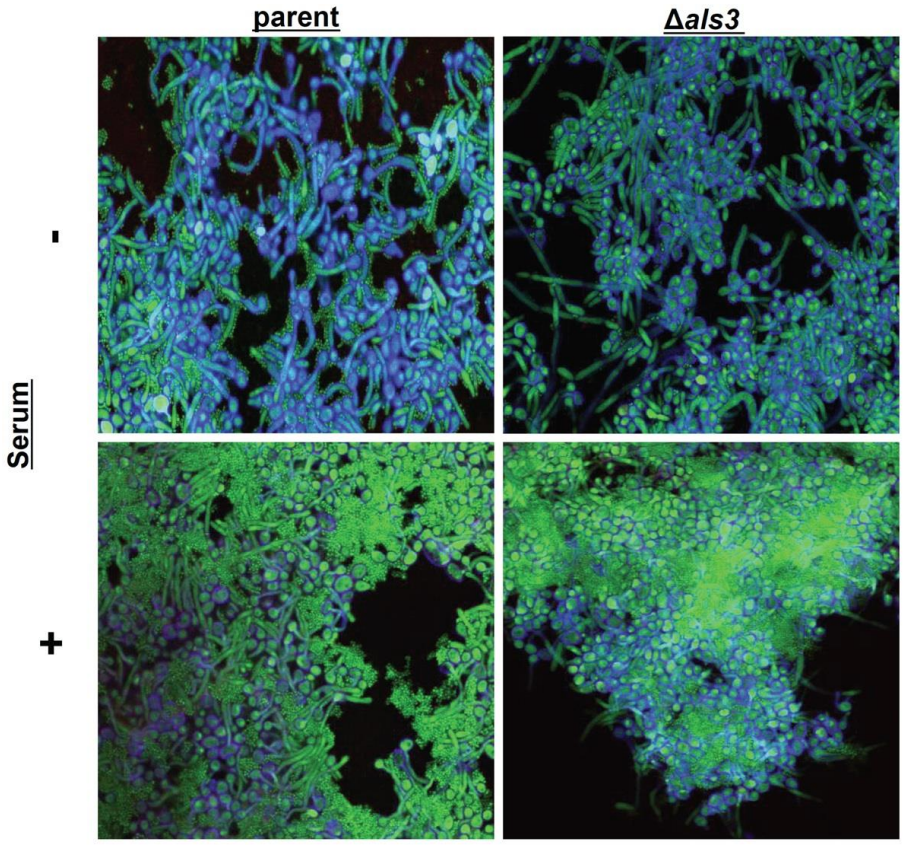

Figure S1. Effect of serum on S*. aureus* adherence to *C. albicans* parent and *als3* mutant strains. Polymicrobial biofilms were formed in the absence and presence of 25% serum, stained with a combination of calcofluor white (blue) / Syto9 (green) and imaged by CSLM. Images demonstrate that in the absence of serum, *S. aureus* adheres only to the hyphae of the parent strain whereas in the presence of serum, *S. aureus* was also able to adhere to the Δ*als3* mutant.

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Chapter 5

Evaluation of adhesion forces of *Staphylococcus aureus* along the length of *Candida albicans* hyphae

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ABSTRACT

Candida albicans is a human fungal pathogen, able to cause both superficial and serious, systemic diseases and is able to switch from yeast cells to long, tube-like hyphae, depending on the prevailing environmental conditions. Both morphological forms of *C. albicans* are found in infected tissue, often in combination with *Staphylococcus aureus*. Although bacterial adhesion to the different morphologies of *C. albicans* has been amply studied, possible differences in staphylococcal adhesion forces along the length of *C. albicans* hyphae have never been determined. In this study, we aim to verify the hypothesis that the forces mediating *S. aureus* NCTC8325-4^{GFP} adhesion to hyphae vary along the length of *C. albicans* SC5314 and MB1 hyphae, as compared with adhesion to yeast cells. *C. albicans* hyphae were virtually divided into a "tip" (the growing and therefore youngest part of the hyphae), a "middle" and a so-called "head" region (the yeast cell from which germination started). Adhesion forces between *S. aureus* NCTC8325-4^{GFP} and the different regions of *C. albicans* SC5314 hyphae were measured using atomic force microscopy. Strong adhesion forces were found at the tip and middle regions of *C. albicans* hyphae (-4.1 nN and -4.0 nN, respectively), while much smaller adhesion forces were measured at the head region (-0.3 nN). Adhesion forces exerted by the head region were comparable with the forces arising from budding yeast cells (-0.5 nN). A similar regional dependence of the staphylococcal adhesion forces was found for the clinical isolate involved in this study, *C. albicans* MB1. This is the first time that differences in adhesion forces between *S. aureus* and different regions of *C. albicans* hyphae have been demonstrated on a quantitative basis, supporting the view that the head region is different from the remainder of the hyphae. Notably it can be concluded that the properties of the hyphal head region are similar to those of budding yeast cells. These novel findings provide new insights in the intricate interkingdom interaction between *C. albicans* and *S. aureus*.

INTRODUCTION

Candida albicans is an opportunistic human pathogen and the leading cause of a wide range of human fungal infections. *C. albicans* is a polymorphic fungus and either grows as a unicellular budding yeast cell or in a filamentous, (pseudo)hyphal form, depending on environmental conditions, such as temperature, pH or presence of chemical stimuli such as serum components or N-acetylglucosamine [1-3]. The ability to switch between different morphologies is important for *C. albicans* virulence [4, 5]. It is presumed that yeast cells facilitate dissemination to target organs, whereas hyphae play a role in further tissue invasion due to the ability to adhere to and pierce host epithelial and endothelial cells, damaging them through the release of hydrolytic enzymes and initiate candidiasis [5-7]. *C. albicans* morphological plasticity also plays an important role in biofilm formation and maturation. *C. albicans* mutants unable to perform morphological switches can develop only rudimentary biofilms, that are structurally less stable than wild type biofilms [8-10].

C. albicans co-exists with a highly diverse bacterial flora in various sites of the human body, resulting in mixed species biofilms [11, 12]. For survival and reproductive success, interacting microorganisms in polymicrobial communities are involved in antagonistic or synergistic relationships. *C. albicans* is often co-isolated with *Pseudomonas aeruginosa* during catheter-associated infections or infections of patients suffering from cystic fibrosis and burn wounds [13,14]. *P. aeruginosa* can specifically adhere to *C. albicans* hyphae but not to yeast cells, which leads to rapid lysis and death of hyphae through a currently unidentified mechanism [15, 16]. *C. albicans* and *Streptococcus gordonii* on the other hand, form a synergistic partnership since these streptococci enhance *C. albicans* filamentation, whereas *C. albicans* can stimulate streptococcal biofilm formation on different kind of surfaces [17]. Klotz et al. [18] showed that in approximately

20% of bloodstream infections, *C. albicans* was co-isolated in conjunction with *Staphylococcus aureus*. Moreover, *C. albicans* and *S. aureus* are able to form complex polymicrobial biofilms on various mucosal surfaces, and within a biofilm *S. aureus* is mostly associated with hyphal cells and not with yeast cells [19, 20]. Interestingly, co-infection of mice with *C. albicans* and *S. aureus* demonstrated a synergistic effect, resulting in increased mice mortality [21, 22]. Furthermore, recent *in vitro* and *in vivo* studies demonstrated that *S. aureus* may use adhesion to *C. albicans* hyphae to become invasive. Using an *ex vivo* murine tongue model, it was shown that oral co-colonization by *C. albicans* and *S. aureus* led to penetration of tongue tissue by hyphae with adhering *S. aureus* [23].

Atomic Force Microscopy (AFM) is a state-of-the-art technique that allows recording of the actual adhesion force that occurs between a bacterium and *C. albicans* (see Figure 1A). AFM has recently been applied to identify the nature of the adhesion forces between *P. aeruginosa* and *C. albicans* [24]. Bacterial adhesion to hyphae was always accompanied by strong adhesion forces, but did not occur to yeast cells. Poisson analyses of adhesion forces indicated that the outermost mannoprotein-layer on hyphal surfaces created favorable acid–base conditions for adhesion, allowing close approach of *P. aeruginosa.* Removal of these proteins caused unfavorable acid–base conditions, preventing adhesion of *P. aeruginosa*. Despite the notable importance of *C. albicans* morphological plasticity for bacterial-fungal interaction, possible differences in bacterial adhesion forces along the length of *C. albicans* hyphae have never been determined. Hyphae grow in a linear mode, with the tip of the hyphae representing the youngest part and the region closer to the original germinating yeast cell being the oldest. Here we hypothesize, that these differences along the length of a hypha may impact the adhesion forces with bacteria. The aim of this paper is to verify this hypothesis. To this end, we virtually divided (see Figure 1B)

C. albicans hyphae into a "tip" (the growing end of the hyphae), a "middle" and a so-called head region (the yeast cell from which germination started) and measured actual adhesion forces that occur between these hyphal regions of two different *C. albicans* strains and a *S. aureus* strain using AFM.

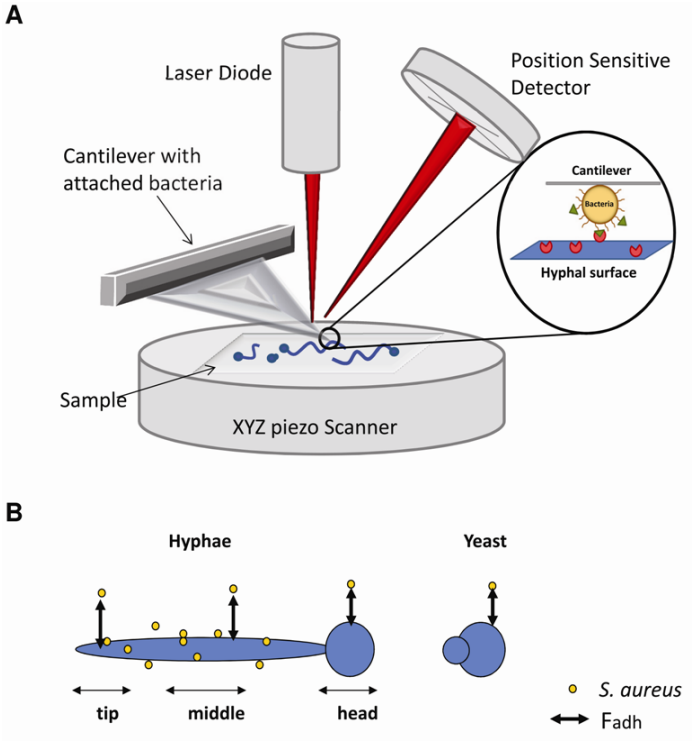

Figure 1 Schematic illustration of the principle of atomic force microscopy and definition of different hyphal regions.
(A) Schematic presentation of AFM set-up. A sample with attached *C. albicans* cells is positioned by a xyz piezo scanner, while a bacterium attached to a tipless AFM cantilever is brought into contact with the hyphal surface. The deflection of the cantilever upon retract is a measure of the adhesion forces between a bacterium and the hyphal surface and is detected by an optical laser. The laser beam is focused on the very end of the cantilever and reflected onto a position sensitive detector from which the adhesion forces can be calculated, provided the mechanical properties of the cantilever are known.
(B) Schematic indication of the different hyphal regions defined for bacterial-hyphal adhesion force measurements.

MATERIALS AND METHODS

Strains, growth conditions and harvesting

C. albicans SC5314 (a commonly used, wild type reference strain), *C. albicans* MB1 (a biofilm-associated, clinical isolate [25]) and bacterial strain *S. aureus* NCTC8325-4 (wild type) were used. To generate green fluorescent protein (GFP)-expressing *S. aureus* NCTC8325-4, pMV158GFP [26] was introduced into competent bacterial cells by electroporation [27]. Selection of subsequent transformants was performed on tryptone soya broth with 1.5% bactoagar (TSB, Oxoid, Basingstoke, UK) plates containing 10 µg/ml tetracycline. *S. aureus* NCTC8325-4 that received pMV158GFP (*S. aureus* NCTC8325-4^{GFP}) showed constitutive GFP expression that could be visualized using fluorescence microscopy.

Strains were grown on TSB agar plates, supplemented with tetracycline when appropriate. Single colonies were inoculated in 5 mL TSB for bacterial pre-cultures or 5 ml yeast nitrogen base acids *(*YNB; Difco, Sparks, USA) pH 7, containing 0.5% D-glucose for *C. albicans* pre-cultures. *S. aureus* was routinely grown at 37°C while *C. albicans* was grown at 30°C to prevent hyphal formation for 24 h with rotation (150 rpm) and used to inoculate a main culture (1:50 dilution of pre-culture). Main bacterial cultures were grown for an additional 18 h under the same conditions. *C. albicans* hyphae were induced by growing a culture (1:50 dilution) for 4 h with rotation (150 rpm) at 37°C. Hyphal formation was obtained at 90-95% efficiency under these conditions, as confirmed by phase contrast microscopy. Main cultures were harvested by centrifugation for 5 min at 6,250 x *g* and 14,800 x *g* for *S. aureus* and *C. albicans*, respectively, followed by two washes with phosphate buffered saline (PBS: 10 mM potassium phosphate, 0.15 M sodium chloride, pH 7) and resuspended in PBS.

Adhesion of staphylococci to hyphae and yeast using fluorescence microscopy

Adhesion of *S. aureus* NCTC8325-4^{GFP} to *C. albicans* in its hyphal morphology was verified using fluorescence microscopy (Leica DM4000B, Heidelberg, Germany). Hyphal formation was induced by growth at 37°C in 12 wells plate (Costar, Corning Inc., NY, USA). After 4 h of hyphal formation, wells were washed once with PBS. Bacteria were added to a final optical density measured at 600 nm (OD_{600}) of 0.1 in PBS. After 3.5 h of co-incubation with staphylococci at 37°C under static conditions, wells were gently washed two times with PBS and *C. albicans* hyphae were counter-stained with Calcofluor White (35 µg/ml, 15 min at room temperature), known to bind to chitin-rich areas of the fungal cell wall. Afterwards, images were taken at five randomly chosen locations in the wells using a 40x water immersion objective using filter sets for GFP and UV. All experiments were performed in triplicate with separately grown cultures.

Staphylococcal adhesion forces along hyphae using atomic force microscopy

Adhesion forces between *S. aureus* NCTC8325-4^{GFP} and hyphae were measured at room temperature in PBS using an optical lever microscope (Nanoscope IV, Digital Instruments, Woodbury, NY, USA) as described before [24]. Briefly, *C. albicans* was immobilized on glass slides (Menzel, GmbH, Germany), coated with positively charged poly-L-lysine. A fungal suspension was deposited onto the coated glass and left to settle at room temperature for 20 min. Non-adhering cells were removed by rinsing with demineralized water and the slide was kept hydrated prior to AFM analysis in phosphate buffer. To create a bacterial probe, *S. aureus* was immobilized onto poly-L-lysine treated tipless “V”-shaped cantilevers (DNP-0, Veeco Instruments Inc., Woodbury, NY, USA). Bacterial probes were freshly prepared for each experiment.

For each bacterial probe, force curves were measured after different bond-maturation times up to 60 s on the same, randomly chosen spot on a hyphal or yeast cell with a z-scan rate of less than 1 Hz. To ensure that no bacteria detached from the cantilever during the experiment, control force-distance curves were made with 0 s contact time after each set of measurements. Whenever the "0 s contact time" forces measured deviated more than 0.5 nN from the initial measurement, a bacterial probe was considered damaged and replaced. For each combination of a bacterial strain and fungal–coated glass surface, five different probes were employed on average and the number of bacterial probes used depended on the outcome of the control measurements. Calibration of each cantilever was done using the thermal tuning method (Nanoscope V6.13r1), yielding a range of spring constants from 0.03 to 0.06 (N/m).

Statistics

Typically, measured bacterial adhesion forces contained a large spread and were not normally distributed (Shapiro–Wilk test, $P < 0.01$). Hence, data are presented as median and interquartile range. Adhesion forces for different fungus-bacterium pairs were compared using non-parametric analyses (Mann-Whitney test). Differences were considered significant when the P-value was < 0.05.

RESULTS

In order to assess the adhesion of *S. aureus* NCTC8325-4^{GFP} along the length of *C. albicans* hyphae, we used two different fungal strains: *C. albicans* SC5314 and *C. albicans* MB1. Bacterial adhesion to hyphae was visualized with fluorescent microscopy and quantitated by enumeration of adhering bacteria per unit hyphal length (Figure 2). Most bacteria adhered to the tip and middle regions of the

hyphae and adhered only scarcely to the head region of the hyphae or to non-germinating yeast cells (Figure 2C). Note that strictly speaking, a comparison of the number of staphylococci adhering per unit hyphal length may not be directly compared with the number of bacteria adhering to a non-germinating yeast cell. Both *C. albicans* strains showed the same trend, although bacteria adhered to *C. albicans* SC5314 in higher numbers than to the clinical isolate MB1.

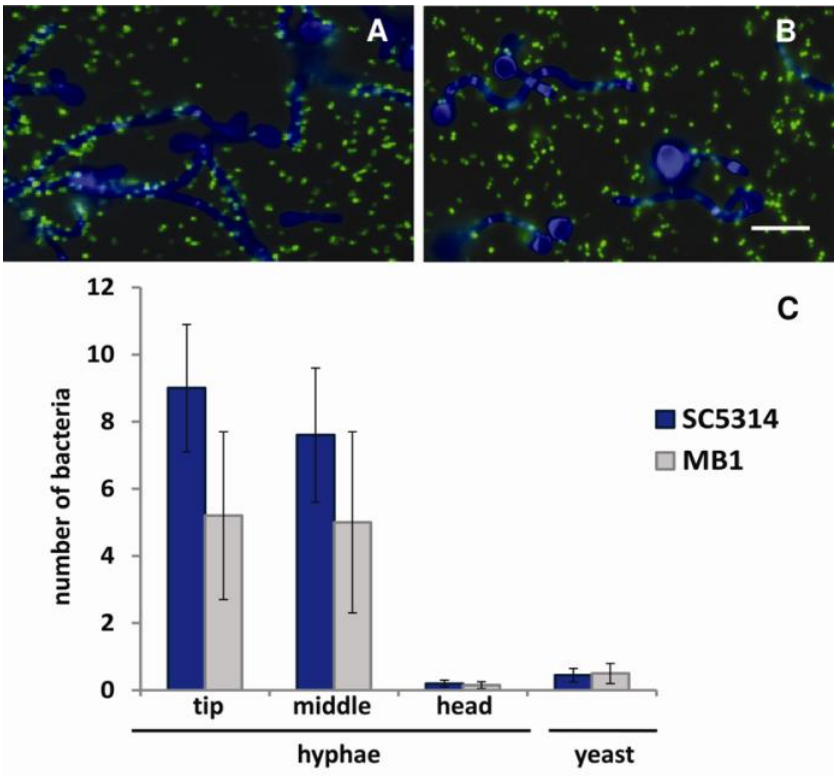

Figure 2 Microscopic analysis of inter-species interaction.
Examples of fluorescent microscopic images and quantitative enumeration of the interaction between *S. aureus* NCTC8325-4^{GFP} and *C. albicans* strains.
(A) *S. aureus* with *C. albicans* SC5314 hyphae.
(B) *S. aureus* with *C. albicans* MB1 hyphae. Scale bar corresponds with 10 µm.
(C) number of *S. aureus* NCTC8325-4^{GFP} adhering per 10 µm length of different regions of *C. albicans* hyphae and yeast cells. Error bars represent SD over three experiments with separately cultured organisms and involving 30 hyphae per bacterium-fungus pair.

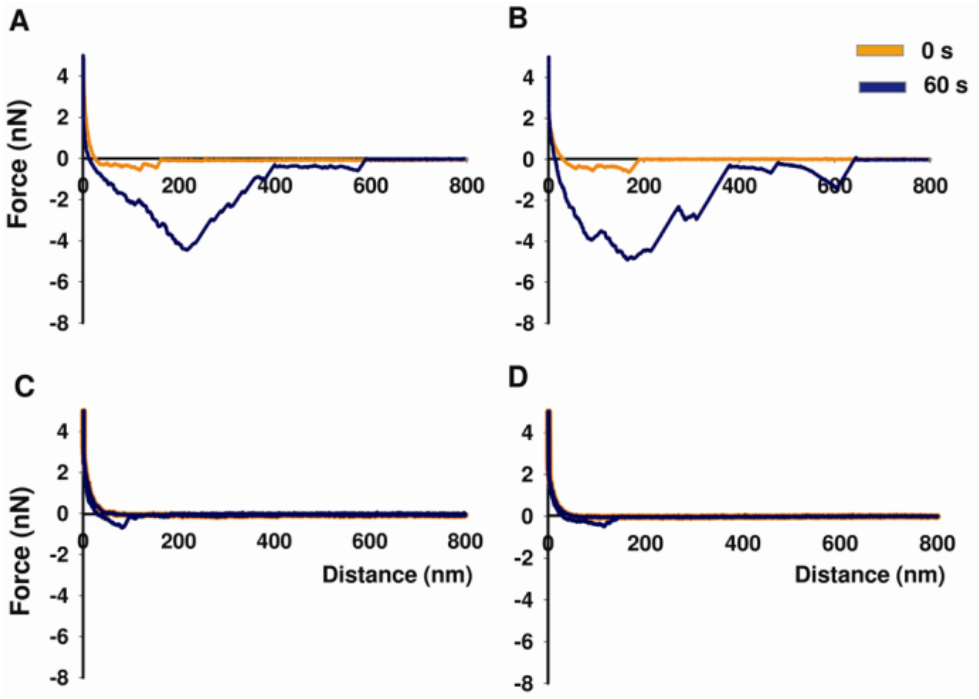

Figure 3 Representative examples of force-distance curves.
Force-distance curves between different *S. aureus* NCTC8325-4^{GFP}-fungus pairs upon initial contact and after 60 s bond-maturation.
(**A**) *C. albicans* SC5314 hyphal tip region;
(**B**) *C. albicans* SC5314 hyphal middle region;
(**C**) *C. albicans* SC5314 hyphal head region;
(**D**) *C. albicans* SC5314 yeast cell.

Adhesion forces between *S. aureus* NCTC8325-4^{GFP} and both *C. albicans* strains along the hyphae were determined using AFM (Figure 1). Figure 3 shows typical examples of force-distance curves of the *S. aureus* probe upon approach and retract from *C. albicans* hyphae and yeast surfaces at initial contact and after 60 s surface delay. Major differences existed in AFM force-distance curves recorded immediately upon contact (0 s) and after a 60 s surface delay between *S. aureus* NCTC8325-4^{GFP} and different hyphal regions and the yeast cell, as summarized in Figure 4. In line with the higher number of bacteria adhering to the tip and middle regions of *C. albicans* hyphae (Figure 2C), stronger adhesion forces (around -4 nN

for SC5314 and around -2 nN for MB1) were recorded after bond-maturation between these regions than for the head regions (around -0.5 nN). However, adhesion forces measured between *S. aureus* NCTC8325-4^{GFP} and both yeast cells remained comparable to the adhesion forces measured to the head region of the hyphae, irrespective of bond-maturation (Figures 4A and 4B). Note that in general, adhesion forces, especially after bond-maturation, were significantly stronger between *S. aureus* and the hyphal regions of *C. albicans* SC5314 than between *S. aureus* and *C. albicans* MB1 hyphal middle and tip regions (compare Figures 4A and 4B).

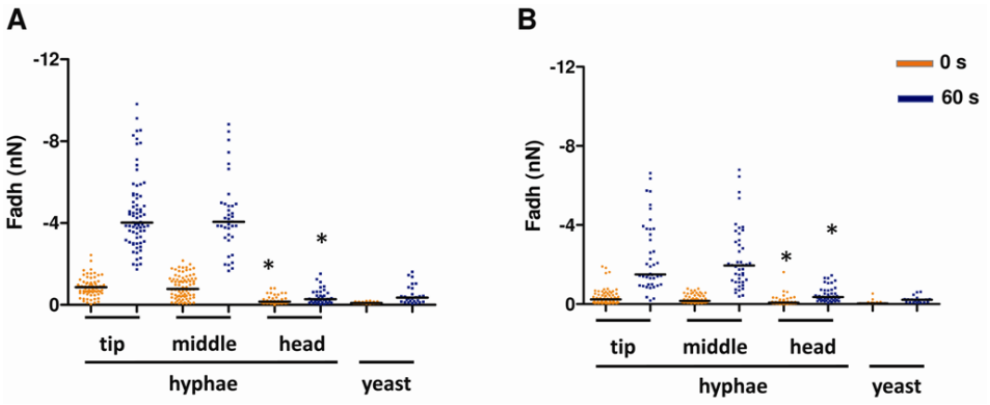

Figure 4 AFM analysis of adhesion forces between *C. albicans* and *S. aureus* NCTC8325-4^{GFP}.
Vertical scatter bars of adhesion forces between *S. aureus* NCTC8325-4^{GFP} and different *C. albicans* strains and morphologies.
(A) Different hyphal regions and yeast cells of *C. albicans* SC5314.
(B) Different hyphal regions and yeast cells of *C. albicans* MB1.
Each data point corresponds to a single force-distance curve recorded between a bacterium and a hypha. Median force values are indicated with a line. Statistically significant differences in adhesion forces ($p < 0.05$; Mann-Whitney test) of bacteria with the hyphal head region *versus* the middle or tip region are indicated by an asterisk.

DISCUSSION

In this study, we hypothesized that *S. aureus* adhesion may vary along the length of *C. albicans* hyphae. To this end, our study was designed to determine the

actual physical interaction between *S. aureus* and hyphae, contingently divided into three regions, i.e. a head, middle and tip region. *S. aureus* adhered in highest numbers to the middle and tip regions of the hyphae and adhered hardly to the head region and yeast cells. In order to give new insights into this intriguing interaction, we measured staphylococcal adhesion forces directly and found that adhesion forces experienced by *S. aureus* varied along the length of *C. albicans* hyphae and were lowest in the head region of hyphae. Importantly, staphylococcal adhesion to the hyphal head region compared well with adhesion to budding yeast cells, which means that the properties of the cell wall, with respect to bacterial adhesion, remain the same for the yeast cell and head region of hyphae upon morphological change. Interestingly, electron microscopy showed that during germination, the yeast cell wall changes its morphology at the site of hyphae initiation and further formation of the germ tube requires extensive cell wall modification [28, 29]. The germ-tube cell wall was not only almost two times thinner than the cell wall of the parental yeast [28, 29], but also much more hydrophobic (water contact angle 107 degrees) than yeast cells (water contact angle 25 degrees) [30]. Hydrophilicity of the yeast cells is caused by the presence of mannoproteinaceous, hydrophilic, surface fibrils. Such fibrils are long, evenly spaced, radiating and mask hydrophobic proteins [31].

The biochemical composition of the cell wall of hyphae and yeast cells of *C. albicans* has been investigated extensively [32, 33]. The *C. albicans* cell wall consists of two main layers: an outer layer of mannoproteins and an inner one that is composed of skeletal polysaccharides, such as chitin and β-1,3-glucans which confer strength and shape [32-34]. Although the basic cell wall components of *C. albicans* remain the same for hyphal and yeast cells, the amount and exposure of polysaccharides, as well as its surface proteome differ significantly [33-35]. For example, the amount of chitin in the hyphal cell wall is 3-5 times

more than in the yeast cell wall, which could be relevant for the interaction with the host's immune system [36]. Expression of a number of hypha-specific cell wall proteins, like agglutinin-like sequence 3 (Als3) protein, is up-regulated during the yeast-hyphae switch [35,37,38]. Als3 is specifically recognized by *Streptococcus gordonii* and allowed bacteria to adhere to the hyphae [39] and is also involved in adhesion of *S. aureus* to *C. albicans* hyphae [23]. Interestingly, Als3 protein was localized exclusively along complete hyphae and was not observed in the head region of hyphae nor in yeast cell walls [40]. This is in line with the current observation that there is no significant difference in adhesion forces between *S. aureus* and the relatively young tip region compared to older regions of the hypha.

Staphylococcal adhesion forces varied within the two *C. albicans* strains involved in this study. This effect can possibly be explained by the differential expression of cell wall associated proteins, e.g. proteins belonging to the Als family. These proteins are recognized as amyloid proteins and able to rearrange to form β-sheets, depending on environmental conditions and the strain of *C. albicans* involved. [37, 38, 41]. Agglutinin-like sequence 3 (Als3p) is known to play a major role in the adherence process between *C. albicans* hyphae and *S. aureus* [23] and we speculate that differences in the density of Als3p along on hyphae between *C. albicans* SC5314 and MB1 account for the different adhesion forces measured with *S. aureus*. This speculation is supported by the increases in adhesion forces observed after 60 s surface delay, that may correspond to unzipping and rearrangement of a β-sheet-rich amyloid fibres [42].

Although bacterial adhesion to the different morphologies of *C. albicans* has been amply studied, possible differences in staphylococcal adhesion along the length of *C. albicans* hyphae have never been determined. Collectively, the findings generated from this study quantified *S. aureus* - *C. albicans* interactions

and demonstrated that the head region of the hyphae is different from other hyphal regions. Therewith this study combines microbiology and physical-chemistry to yield a better understanding of the fast developing field of interkingdom interactions.

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Chapter 6

Exchange of adsorbed serum proteins

during adhesion of *Staphylococcus aureus*

to an abiotic surface and *Candida albicans* hyphae

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ABSTRACT

Staphylococcus aureus and *Candida albicans* are the second and third most commonly isolated microorganisms in hospital-related-infections, that are often multi-species in nature causing high morbidity and mortality. Here, adhesion forces between a *S. aureus* strain and abiotic (tissue-culture-polystyrene, TCPS) or partly biotic (TCPS with adhering hyphae of *C. albicans*) surfaces were investigated in presence of fetal-bovine-serum or individual serum proteins and related with staphylococcal adhesion. Atomic-force-microscopy was used to measure adhesion forces between *S. aureus* and the abiotic and biotic surfaces. Adsorption of individual serum proteins like albumin and apo-transferrin to abiotic TCPS surfaces during 60 min, impeded development of strong adhesion forces as compared to fibronectin, while 60 min adsorption of proteins from fetal-bovine-serum yielded a decrease in adhesion force from -5.7 nN in phosphate-buffered-saline to -0.6 nN. Adsorption of albumin and apo-transferrin also decreased staphylococcal adhesion forces to hyphae as compared with fibronectin. During 60 min exposure to fetal-bovine-serum however, initial (5 min protein adsorption) staphylococcal adhesion forces were low (-1.6 nN), but strong adhesion forces of around -5.5 nN were restored within 60 min. This suggests for the first time that in whole fetal-bovine-serum exchange of non-adhesive proteins by fibronectin occurs on biotic *C. albicans* hyphal surfaces. No evidence was found for such protein exchange on abiotic TCPS surfaces. Staphylococcal adhesion of abiotic and biotic surfaces varied in line with the adhesion forces and was low on TCPS in presence of fetal-bovine-serum. On partly abiotic TCPS, staphylococci aggregated in presence of fetal-bovine-serum around adhering *C. albicans* hyphae.

INTRODUCTION

Microorganisms form biofilms in most natural environments, including different sites of the human body [1, 2]. In the human body, biofilms can develop on abiotic surfaces, like on biomaterial implants or devices, as well as on living tissues [1]. Biofilms in the human body can either be single-species, or consist of a combination of different species, including fungi and bacteria. In intravascular catheter-related infections for instance, biofilms comprise combinations of *Streptococcus* spp*, Staphylococcus aureus,* or Gram-negative bacilli with *Candida albicans* [2]. *C. albicans,* a unicellular polymorphic opportunistic human fungal pathogen, can form biofilms on a wide variety of polymers and constitutes one of the leading causes of nosocomial bloodstream infections [3]. Klotz *et al.* [4] showed that in approximately 20% of bloodstream infections, *C. albicans* was co-isolated with *S. aureus. S. aureus* is a Gram-positive coccus and an opportunistic pathogen that has rapidly developed antibiotic resistance to a number of clinically important antibiotics. It is responsible for a wide range of diseases, from relatively harmless skin infections to potentially fatal systemic disorders [5, 6].

Organisms in multi-species biofilms often take benefit from each other's presence. For example, lactate excreted by oral streptococci, is used as a carbon source by *C. albicans* [7, 8]. *C. albicans*, on the other hand, may stimulate streptococcal growth and colonization by reducing the oxygen tension to levels preferred by streptococci [9, 10]. In mixed-species biofilms with *S. epidermidis* or *S. aureus*, *C. albicans* protects staphylococci against antibiotics, such as vancomycin [11, 12]. Vice versa, slime-producing *S. epidermidis* protects *C. albicans* against fluconazole [13]. The mechanism mediating staphylococcal tolerance for vancomycin by *C. albicans* is not clear yet and requires further investigation, although it has been suggested that direct physical contact between the species and *C. albicans* extracellular matrix may play an important role [11,

12]. Interestingly, co-infection of mice with *C. albicans* and *S. aureus* demonstrated a synergistic effect on mouse mortality [14].

Although *S. aureus* can form biofilms on abiotic surfaces that come into contact with blood, *S. aureus* has difficulty forming biofilms on its own, especially in the presence of serum [11, 15]. In contrast, serum increases *C. albican*s biofilm formation on polystyrene surfaces *in vitro* [16] and serves as one of the most well-known inducers of yeast-into-hyphae transition [17, 18]. Moreover, *S. aureus* biofilm formation on biotic surfaces, like on hyphae of *C. albicans* was enhanced in serum-supplemented media [11]. Serum consists of various components, that may all affect microbial adhesion in different ways. Bovine serum albumin (BSA) has been described to have an inhibitory effect on adhesion of *Pseudomonas* spp. to polystyrene [19]. Apo-transferrin, the iron-free form of transferrin (a monomeric serum glycoprotein capable of binding iron ions) is known to suppress adhesion of a variety of microbial pathogens to abiotic materials, such as polystyrene, polymethylmethacrylate and silicone rubber [20, 21]. Adsorption of fibronectin and vitronectin on the other hand, can promote adhesion of *Staphylococcus* spp. to implanted abiotic materials through specific ligand-receptor interactions [22-24].

Microbial adhesion and colonization are mediated by attractive forces between biotic or abiotic surfaces and the organisms, that can be quantitatively assessed by atomic force microscopy (AFM) [25]. Bacterial adhesion and (co)aggregation have both been demonstrated to be sensitive to minor differences in adhesion forces between strains [26, 27]. It has even been argued that a beneficial effect of cranberry juice on adhering urogenital pathogens could be attributed to a reduction in adhesion force between *Escherichia coli* and a silicon nitride AFM tip upon adsorption of cranberry juice components from stronger than -0.5 nN to less than -0.5 nN [28]. Moreover, strong adhesion forces

of up to -8.6 nN have been observed between *Pseudomonas aeruginosa* strains and hyphae of *C. albicans* that were absent for yeast [29].

The aim of this study was to determine the role of adsorbed serum proteins on *S. aureus* adhesion forces in the initial adhesion to an abiotic (tissue culture polystyrene, TCPS) or partly abiotic surface (TCPS with adhering hyphae of *C. albicans*).

MATERIALS AND METHODS

Strains, growth conditions and harvesting

C. albicans SC5314 was routinely grown for 48 h at 30°C on tryptone soya broth (TSB, Oxoid, Basingstoke, UK) plates with 1.5% bactoagar from glycerol stocks stored at -80°C. Single colonies were used to inoculate 5 ml yeast nitrogen base (YNB; Difco, Sparks, USA) pH 7, containing 0.5% D-glucose for precultures of *C. albicans*. For AFM experiments *C. albicans* germ-tubes, representing young hyphae, were induced by growing a culture (1:50 dilution of a preculture) for 4 h with rotation (150 rpm) at 37°C [30]. Germ-tube formation was obtained at 90-95% efficiency under these conditions, as confirmed by phase contrast microscopy. Hyphae were harvested by centrifugation for 5 min at 14,800 x *g* followed by two washes with phosphate buffered saline (PBS; 10 mM potassium phosphate buffer, 0.15 M sodium chloride, pH 7). After washing, the hyphae were resuspended in PBS.

S. aureus NCTC8325-4 was routinely cultured for 24 h at 37°C on TSB agar plates from glycerol stocks stored at -80°C. Single colonies were used to inoculate 5 ml TSB for precultures of *S. aureus* and were grown for 24 h with rotation (150 rpm) and used to inoculate a main culture (1:50 dilution). The main culture was grown for an additional 18 h under the same conditions. Bacteria were harvested by centrifugation for 5 min at 6,250 x *g* followed by two washes with PBS, after

which staphylococci were suspended in PBS to an optical density OD_{600} of 0.1 measured at 600 nm.

To generate green fluorescent protein (GFP)-expressing *S. aureus* NCTC8325-4, pMV158GFP [31] was introduced into competent bacteria by electroporation, as described by Li *et al*. [32]. Subsequent transformants were selected on TSB agar plates containing 10 µg/ml of tetracycline. *S. aureus* NCTC8325-4^{GFP} was grown in TSB media containing 10 µg/ml tetracycline, as described above and GFP expression in *S. aureus* NCTC8325-4^{GFP} carrying pMV158GFP was confirmed using fluorescence microscopy.

Adhesion of staphylococci to TCPS and hyphae in absence and presence of serum proteins

C. albicans preculture (1:100 dilution) was used to initiate hyphal adhesion in 12 wells TCPS plates (Costar, Corning Inc., NY, USA). The wells were incubated for 4 h at 37°C without rotation. After 4 h of growth, wells were washed once with PBS in order to remove non-adherent cells. Subsequently, staphylococcal suspension was added to the wells and bacteria and hyphae of *C. albicans* were allowed to adhere under static conditions for the next 3.5 h either in PBS, or PBS supplemented with 50% fetal bovine serum (FBS; Sigma, USA), 50 µg/ml BSA (Sigma, USA), 5 µg/ml apo-transferrin (apo-Tf; Sigma, New-Zealand) or 20 µg/ml fibronectin (Fn; Sigma, USA). After 3.5 h, wells were gently washed three times with PBS and images were taken either with phase contrast or fluorescence microscopy (Leica DM4000B, Heidelberg, Germany) at five randomly chosen locations in the wells, using a 40x water immersion objective.

Adhesion of *S. aureus* NCTC8325-4^{GFP} to TCPS and TCPS with adhering *C. albicans* hyphae was also evaluated using a Fluorstar OPTIMA spectrophotometer (BMG LabTech, Offenburg, Germany) by excitation at 485 nm and detection at

520 nm. To this end, *C. albicans* hyphae were stained for 15 min at room temperature with calcofluor white (35 µg/ml in PBS), known to bind to chitin-rich areas of the fungal cell wall. The emission wavelength of calcofluor white is 450 nm and excitation occurs around 350 nm. Adhesion experiments were performed in triplicate with separately grown cultures.

Staphylococcal adhesion forces

Staphylococcal adhesion forces were measured at room temperature either in PBS or in PBS supplemented with 50% FBS, 50 mg/ml BSA, 5 mg/ml apo-transferrin or 20 µg/ml fibronectin, using AFM (Nanoscope IV, Digital Instruments, Woodbury, USA), as described before [29].

Briefly, to create a bacterial probe, *S. aureus* was immobilized onto poly-L-lysine treated tipless "V"-shaped cantilevers (DNP-0, Veeco Instruments Inc., Woodbury, NY, USA). Bacterial probes were freshly prepared for each experiment. *C. albicans* hyphae were immobilized on glass slides (Menzel, GmbH, Germany) coated with positively charged poly-L-lysine. To this end, a fungal suspension was deposited onto the coated glass and left to settle at room temperature for 20 min. Non-adhering cells were removed by rinsing with demineralized water and the slide was kept hydrated in PBS. For each staphylococcal probe, force curves were measured after different bond-maturation times up to 60 s on the same, randomly chosen spot on a fungal cell with z-scan rates of less than 1 Hz. To ensure that no staphylococci detached from the cantilever during the experiment or to exclude cell surface contamination from contact with hyphae, force-distance curves were made with the staphylococcal probe *versus* TCPS using 0 s contact time prior to and after each set of measurements. Whenever the adhesion force of a probe *versus* TCPS differed more than 0.5 nN from the initial value, a probe was discarded. On average, for each condition, five different probes were used,

depending on the outcome of the control measurements. Calibration of each cantilever was done using the thermal tuning method (Nanoscope V6.13r1), yielding a range of spring constants from 0.04 to 0.06 (N/ m).

Staphylococcal adhesion forces were measured after 60 min exposure of the surfaces in PBS or PBS supplemented with FBS or different, individual serum proteins (BSA, apo-transferrin or fibronectin). In addition, staphylococcal adhesion forces were measured as a function of exposure time to PBS supplemented with FBS up to 60 min.

Statistics

The Student t-test was used to analyze the significance of differences between experimental groups. Differences between data sets were considered significant when the P-value was < 0.05. Typically bacterial adhesion forces were not normally distributed (Shapiro–Wilk test, $P < 0.01$) and contained a large spread. Hence, they are presented as median and interquartile range. Adhesion forces for different conditions were compared using non-parametric analyses (Mann-Whitney test) [29]. Differences were considered significant when the P-value was < 0.05.

RESULTS

In absence of adhering *C. albicans* hyphae, *S. aureus* NCTC8325-4 in PBS adhered to abiotic TCPS surfaces in high numbers, while a significant reduction was observed when adhesion occurred in the presence of 50% FBS (see Figure 1A). When TCPS was made partly biotic through adhesion of *C. albicans* hyphae, *S. aureus* in PBS adhered to an abiotic TCPS surface in approximately equal numbers as in absence of adhering *C. albicans* hyphae. In presence of 50% FBS, *S. aureus* preferred to form aggregates on the surface of the hyphae, but hardly adhered to

the abiotic TCPS surface (see also Figure 1A). Since adhesion was hard to quantitate by phase-contrast microscopy, experiments were repeated using GFP-producing *S. aureus* NCTC8325-4^{GFP}. Fluorescence microscopic analysis showed that *S. aureus* NCTC8325-4^{GFP} behaved similarly to the wild-type strain with respect to its adhesion to TCPS in absence and presence of adhering *C. albicans* hyphae (compare Figures 1A and 1B). Quantification of GFP-fluorescence due to staphylococcal adhesion (see Figure 1C) showed that in the presence of 50% FBS staphylococci were not able to adhere to the abiotic TCPS in significant numbers. Staphylococcal adhesion to the combined abiotic-biotic surface, i.e. TCPS with adhering *C. albicans* hyphae, was restored in the presence of 50% FBS to similar levels as observed for staphylococcal adhesion to TCPS without FBS. Moreover, presence of individual serum proteins such as BSA and apo-transferrin, but not fibronectin, significantly suppressed staphylococcal adhesion to TCPS and hyphal surfaces (Figure 1C).

AFM was used to measure initial adhesion forces (0 s surface delay) and adhesion forces after 60 s bond-maturation between staphylococci and the different surfaces. In general, bond-maturation increased staphylococcal forces significantly (see Figure 2). In line with the high numbers of staphylococci adhering to TCPS surfaces (Figure 1), strong adhesion forces were observed for *S. aureus* NCTC8325-4 to abiotic TCPS surfaces in PBS (Figure 2A). In contrast, but in line with the reduction in numbers of adhering staphylococci (see Figure 1), staphylococcal adhesion forces to TCPS decreased almost 10-fold from -5.7 nN in PBS to -0.6 nN after 60 min adsorption of proteins from 50% FBS. Interestingly, staphylococcal adhesion forces to biotic hyphal surfaces were not significantly affected by the presence of FBS, although a tendency for increased adhesion forces in the presence of FBS existed (see Figure 2B).

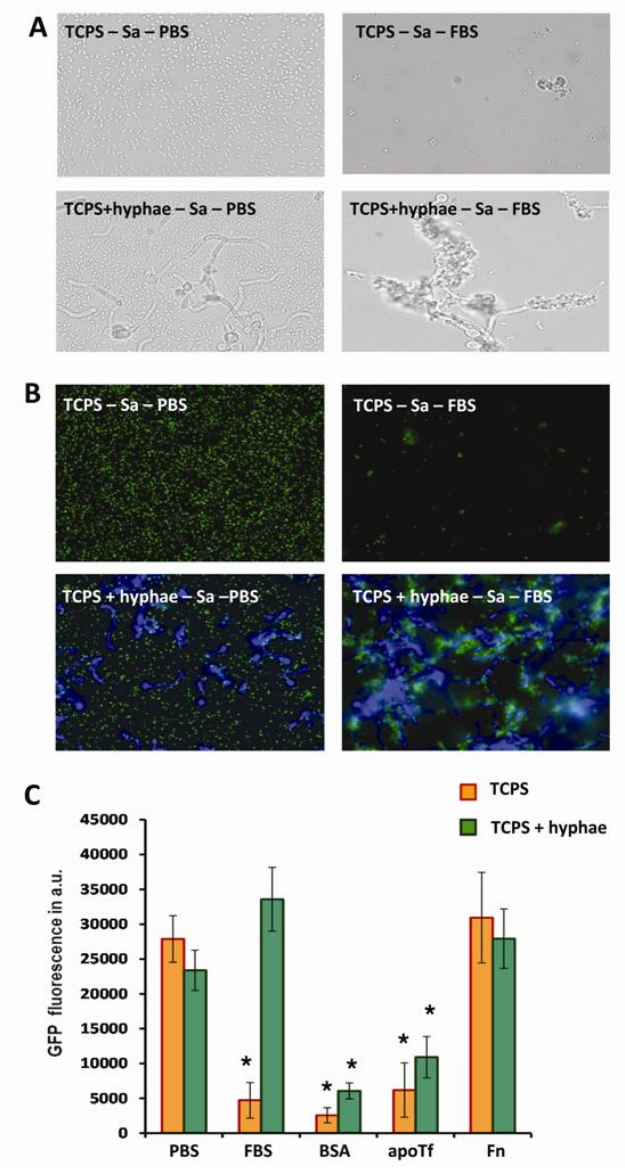

Figure 1 (A) Phase-contrast microscopic images of the initial (3.5 h) adhesion of *S. aureus* NCTC8325-4 (Sa) on a TCPS surface in absence and presence of adhering *C. albicans* SC5314 hyphae in PBS or PBS supplemented with 50% FBS. **(B)** Fluorescence microscopic images of the initial (3.5 h) adhesion of green-fluorescent *S. aureus* NCTC8325-4^{GFP} on a TCPS surface in absence and presence of adhering *C. albicans* hyphae in PBS or PBS supplemented with 50% FBS. Note that hyphae are stained with calcofluor white, yielding blue fluorescence. **(C)** GFP fluorescence due to adhesion by *S. aureus* NCTC8325-4^{GFP} on TCPS and TCPS with adhering *C. albicans* hyphae in PBS, PBS supplemented with 50% FBS and PBS supplemented with different individual serum proteins as BSA (50 mg/ml), apo-transferrin (apoTf; 5 mg/ml) and fibronectin (Fn; 20 µg/ml). Error bars indicate the standard deviations over triplicate experiments with separately cultured organisms. * indicate significant differences ($P < 0.05$) between fluorescence due to adhesion by *S. aureus* NCTC8325-4^{GFP} in PBS *versus* PBS supplemented with different serum proteins.

Figure 2 also shows the effects of three different, individual proteins occurring in FBS on staphylococcal adhesion forces to TCPS (Figure 2A) and hyphae (Figure 2B). Both adsorption of BSA and apo-transferrin decreased staphylococcal adhesion forces to abiotic TCPS and biotic hyphal surfaces as compared to effects of fibronectin adsorption. This suggests that whereas fibronectin alone has the ability to adsorb to both TCPS and hyphal surfaces, when in competition with other serum proteins its exclusively adsorbs to biotic hyphal surfaces.

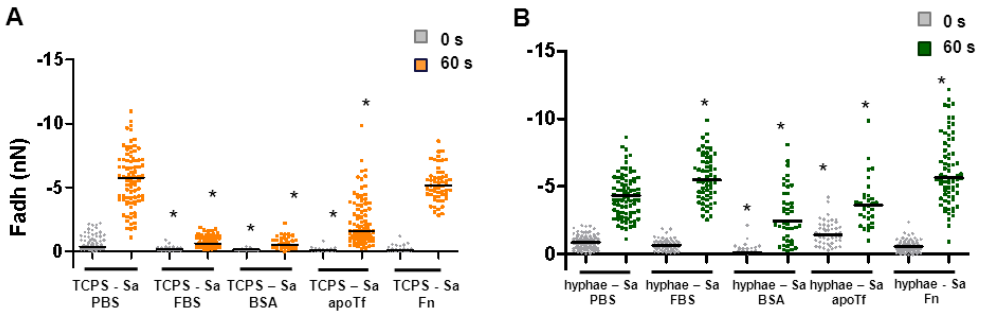

Figure 2 Vertical scatter bars of adhesion forces between *S. aureus* NCTC8325-4 and abiotic TCPS and biotic *C. albicans* hyphal surfaces in PBS, PBS supplemented with 50% FBS and PBS supplemented with different, individual serum proteins. Adhesion forces were taken 60 min after exposure to PBS or protein solutions, with adhesion forcers measured immediately upon bacterial contact with hyphal or TCPS surfaces (0 s) and after 60 s bond-maturation. See Fig. 1 for abbreviations and concentrations of the proteins.
(A) *S. aureus* NCTC8325-4 to abiotic TCPS surfaces.
(B) *S. aureus* NCTC8325-4 to biotic *C. albicans* SC5314 hyphae.
Each data point corresponds to a single force-distance curve recorded between a bacterium and a TCPS or hyphal surface. Median force values are indicated with a line. * indicate statistically significant differences ($P < 0.05$; Mann-Whitney test) in adhesion forces between *S. aureus* NCTC8325-4 and different surfaces in PBS *versus* PBS supplemented with different serum proteins.

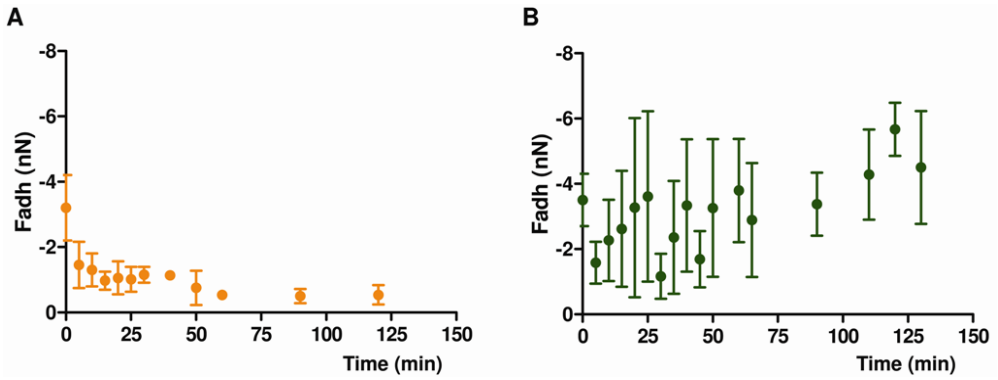

Figure 3 Adhesion forces after 20 s of bond-maturation between *S. aureus* NCTC8325-4 and abiotic TCPS and biotic *C. albicans* hyphal surfaces in 50% FBS as a function of the adsorption time.
(A) *S. aureus* NCTC8325-4 to abiotic TCPS surfaces.
(B) *S. aureus* NCTC8325-4 to biotic *C. albicans* SC5314 hyphae.
Data represent average adhesion forces ± standard deviations over 12 force-distance curves.

In order to verify this hypothesis, we measured staphylococcal adhesion forces as a function of time during exposure to whole FBS (see Figure 3). Adhesion forces on TCPS were initially low, and further decreased during the course of time, while on hyphal surfaces adhesion forces initially decreased within 5 min to low values, but after 50-60 min adhesion forces became stronger again to levels observed after adsorption of fibronectin from a single protein solution. Note that the time-dependent measurement of adhesion forces did not allow us to pursue bond-maturation for 60 s. Instead bond-matured adhesion forces reported in Figure 3 pertain to 20 s surface delay times. This however, does not impede comparison with bond-matured adhesion forces measured after 60 s, as previous studies have indicated that bond-maturation is generally completed within 20 s [29, 33].

DISCUSSION

In this study, the impact of adsorption of serum proteins on adhesion forces and initial adhesion by a *S. aureus* strain to abiotic TCPS and biotic *C. albicans* hyphal surfaces was investigated. Staphylococcal adhesion varied in line with the adhesion forces measured, as summarized in Figure 4.

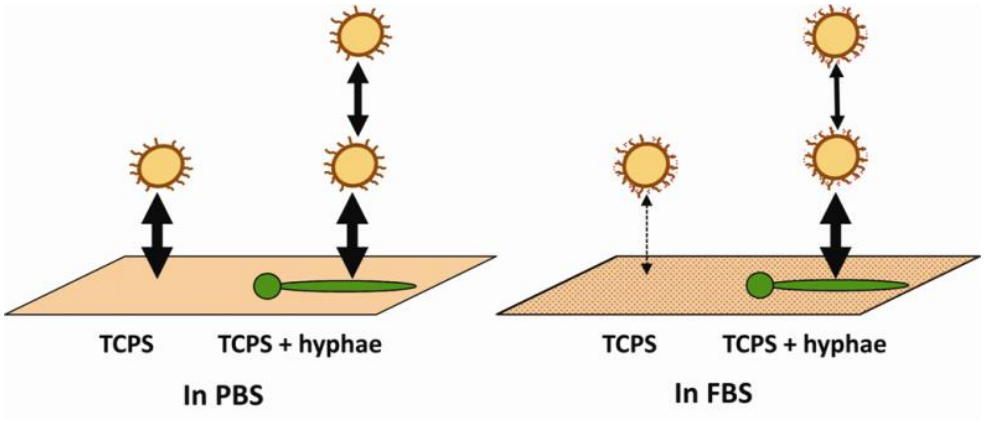

Figure 4 Schematic summary of the adhesion forces between *S. aureus* and biotic (*C. albicans* hyphae) or abiotic surfaces (TCPS) in absence and presence of FBS. In absence of FBS, staphylococci can adhere either to TCPS or hyphal surfaces with similar adhesion forces, but adsorption of serum proteins on TCPS, like albumin and apo-transferrin, impede development of strong adhesion forces. On hyphal surfaces however, these non-adhesive proteins are replaced within 60 min by fibronectin, which restores strong adhesion forces between staphylococci and hyphal surfaces. Note that in order to complement this figure, staphylococcal adhesion forces were measured to each other prior to and after 60 min adsorption of proteins from whole serum, yielding bond-maturated adhesion forces of -2.9 and -1.8 nN, respectively.
The thickness of the arrows symbolizes the magnitudes of the adhesion forces.

Adhesion assays demonstrated that the presence of FBS reduced adhesion of *S. aureus* towards to abiotic TCPS, but not to hyphae. Reduced adhesion of *S. aureus* to abiotic TCPS is in line with previously reported inhibitory effects of serum on initial adhesion and biofilm formation by Gram-positive and Gram-negative

bacterial species on abiotic surfaces [11, 15, 34, 35]. As in previous studies, differences existed in adhesion forces between staphylococci and the different surfaces involved upon initial (0 s) contact and after bond-maturation and usually bond-matured forces are observed within 20 s [36]. This type of bond-maturation is not biological in origin but relates to the removal of water from in between the interacting surfaces, conformational changes of proteins and a general increase in number of contact points [37].

Concurrent with a reduction in staphylococcal adhesion was a reduction in bond-matured adhesion forces between *S. aureus* and TCPS in presence of FBS to levels observed after adsorption of BSA and apo-transferrin from single protein solutions. Since strong adhesion forces were observed after exposure of TCPS to a fibronectin, single protein solution, this demonstrates that adsorption of BSA and apo-transferrin impedes adsorption of fibronectin from whole FBS to TCPS, required to restore strong adhesion forces. As an important new finding from this study, also adsorption of BSA and apo-transferrin from whole FBS reduces staphylococcal forces to hyphal surfaces to levels observed for adsorption of BSA and apo-transferrin from single protein solutions, but after 50-60 min strong adhesion forces arise again, that are comparable to those observed after fibronectin adsorption from a single protein solution. This indicates that fibronectin is able to displace BSA and apo-transferrin from *C. albicans* hyphae, but not from TCPS surfaces.

Adsorption of individual proteins from serum to abiotic surfaces is known to be a highly complicated process with a continuous concentration- and affinity-dependent shift of adsorbed proteins on the surface, (called the “Vroman effect” [38, 39]). High molecular weight proteins usually adsorb more slowly than lower molecular weight proteins. As a result, albumin and other low molecular weight serum proteins adsorb first, but are subsequently displaced over time by higher

molecular weight proteins like fibronectin, adsorbing more irreversibly than smaller ones. The Vroman effect also includes an interesting aspect with respect to the role of hydrophobicity in the exchange of adsorbed proteins on a surface: displacement of low molecular weight proteins proceed towards higher molecular weight proteins predominantly on hydrophilic surfaces, while on more hydrophobic surfaces exchange is almost absent and confined to the initially adsorbing low molecular weight proteins. Our data suggest that BSA and apo-transferrin adsorbed to TCPS cannot be displaced by fibronectin, whereas adsorbed on hyphal surfaces they are displaced by fibronectin. This is at odds with the Vroman displacement series of proteins as valid for abiotic surfaces [29, 40], since TCPS (water contact angle 67 degrees) is more hydrophilic than *C. albicans* hyphae (water contact angle 107 degrees) and can thus be expected to allow greater exchange of low-molecular weight proteins by high molecular weight ones than hyphal surfaces. On abiotic surfaces however, hydrophobicity may not be the only factor controlling protein displacement series and also the presence of specific fibronectin-binding sites on *C. albicans* [41-44] will contribute to displacement of low-molecular weight proteins, that always adsorb first from a multi-component protein solution as is serum due to their faster diffusion.

Summarizing, this study is the first to show non-adhesive proteins, adsorbed from whole serum are displaced by fibronectin over time on biotic *C. albicans* hyphal surfaces. No evidence was found for such protein exchange on abiotic TCPS surfaces, in line with the known “Vroman effect”. This protein exchange results in attractive conditions between staphylococci and biotic *C. albicans* hyphae in serum that are absent on abiotic TCPS surfaces. Hence staphylococci need biotic hyphal surfaces in order to colonize abiotic, hydrophobic surfaces through MSCRAMM (microbial surface components recognizing adhesive matrix molecules) interactions with adsorbed fibronectin

[45, 46], that only become available in serum over time. Moreover, due to the differences in adhesion forces between staphylococci themselves, and between staphylococci and hyphae *versus* TCPS, staphylococci preferentially adhere to hyphae and each other to form aggregates. Such aggregates may constitute a protective environment for staphylococci against environmental attacks, such as by antibiotics [11, 12] and may constitute a simple reason as to why antibiotic tolerance of staphylococci develops in presence of *C. albicans* hyphae.

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Chapter 7

General Discussion

In nature microorganisms tend to form surface-attached communities surrounded by an extracellular matrix, called biofilms. Even though there are known examples of single species biofilms, most biofilms are consisting of multiple species, including bacteria, fungi, protozoa and/or viruses. Mixed bacterial-fungal biofilms are a threat for human health especially in case of immuno-compromised individuals suffering from burn wounds, HIV infection, organ transplantation or patients after X-ray irradiation.

Candida albicans, a polymorphic fungus, is known to be the most prevalent fungus forming pathogenic biofilms and was therefore the main focus of this research project. Although *C. albicans* is known of being capable to form extensive mono-species biofilms causing a wide range of infections, it is often co-isolated in conjunction with other pathogens such as the Gram-negative bacterium *Pseudomonas aeruginosa* and Gram-positive bacterium *Staphylococcus aureus*. Interestingly, these microorganisms are not just simultaneously occupying the same sites of the human body but also pose a complex relationship that is important from a molecular (see Figure 1 for an overview) and clinical perspective.

Though bacterial-fungal interactions within polymicrobial biofilms grasped a lot of attention recently, precise mechanisms of adhesion and signaling involved in their dynamic interaction are still under investigation. The complexity of *in vivo* bacterial-fungal-host interactions can be best exemplified by the interaction between *P. aeruginosa* and *C. albicans*. *P. aeruginosa* can form a thick biofilm on *C. albicans* hyphae and can kill the fungus. Interestingly, *P. aeruginosa* can neither colonize, nor kill yeast cells. Several *in vitro* models of mixed biofilms have been used to investigate this interaction and it was shown that *P. aeruginosa* is able to inhibit *C. albicans* hyphae formation and its metabolic activity. *C. albicans* in its turn secretes farnesol, a quorum sensing molecule, which at low cell density inhibits Pseudomonas quinolone signal production which is required for

the expression of several virulence factors (Figure 1). Although the interaction between *P. aeruginosa* and *C. albicans* is usually pointed out as an example of an antagonistic relationship, in an *in vivo* rat study as well as in clinical studies, it has been shown that pre-colonization of lung tissue or urinary catheters by *C. albicans* increases the risk of developing a *P. aeruginosa* infection.

The pathways of interspecies interaction as outlined in Figure 1 mainly involve signaling molecules that have to diffuse through a liquid phase in order to become effective. This is in line with the general observation that a certain density of bacteria is needed for effective cross-talk to become initiated. At the same time, this suggests that adhesion, i.e. physical interaction between species as in a biofilm, may be a requirement for enhancing an effective interaction, as this brings the organism in closer vicinity to each other. This consideration led to the aim of this thesis: to gain insight into physico-chemical and biological mechanisms involved in interactions between the eukaryotic opportunistic pathogen, *C. albicans* and different bacterial strains.

The importance of physical interaction is elegantly demonstrated in Chapter 6 of this thesis, where it is shown how *C. albicans* hyphae not only assist *S. aureus* in colonizing abiotic surfaces in the presence of serum, but also allow the bacterial strain to form aggregates on their surfaces. This biofilm mode of growth then resists antibiotic penetration through the bacterial biofilm (see Figure 2) which may constitute a simpler reason for antibiotic tolerance than hitherto proposed [2, 3] on the basis of molecular mechanisms.

An *ex vivo* murine tongue model, recently demonstrated that oral co-colonization by *C. albicans* and *S. aureus* led to penetration of tongue tissue by hyphae with adhering *S. aureus,* which resulted in a systemic staphylococcal infection [4].

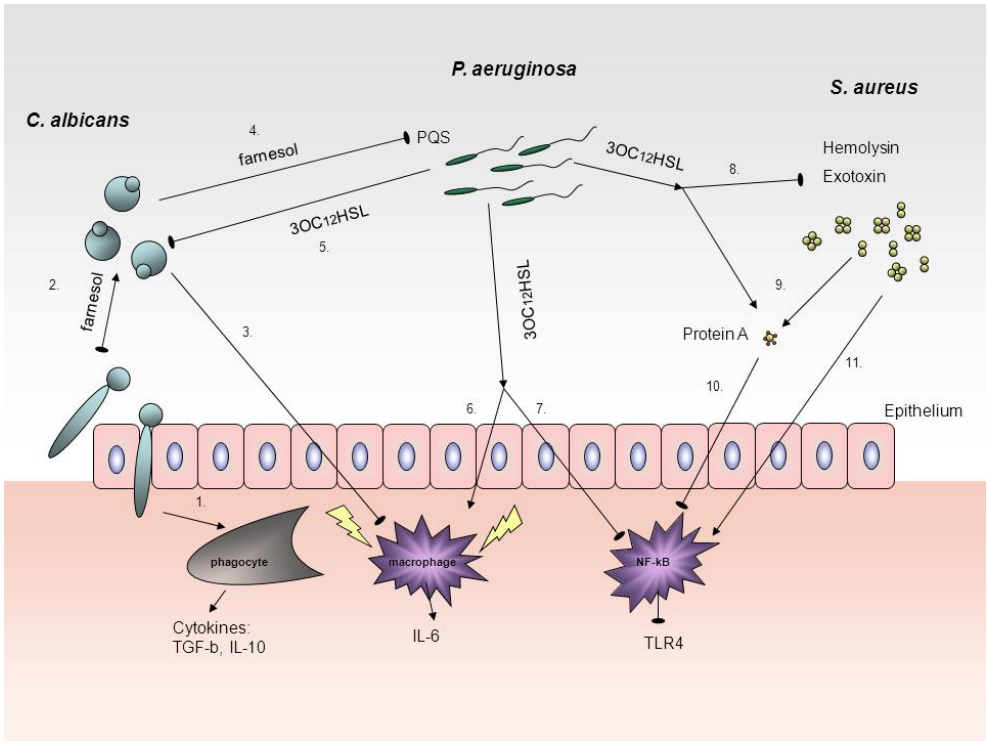

Figure 1 Interspecies interaction during the bacterial-fungal co-infection. Lines ending in arrows indicate induction and lines ending in circles indicate inhibition of the indicated process. *C. albicans*, in the hyphal morphology, induces phagotycosis during inflammation (1). Virulent hyphae can be inhibited and transformed to yeast cells by farnesol, the quorum-sensing molecule secreted by *C. albicans* (2). *Candida* yeast cells prevent macrophages induction (3) and render *C. albicans* invisible for the immune system. *C. albicans* coexisting with *P. aeruginosa* exerts a double-sided reaction; farnesol inhibits *Pseudomonas* quorum-sensing production (4), whereas $3OC_{12}HSL$ secreted by *P. aeruginosa* prevents *C. albicans* hyphae formation without changing the growth rate (5). $3OC_{12}HSL$ sensed by the host induces a pro-inflammatory response by activation of macrophages (6), but it can also give an anti-inflammatory reaction by selectively diminishing the regulation of NF-κB signalling and attenuating TLR4-dependent innate immune responses (7). *P. aeruginosa* $3OC_{12}HSL$ influences *S. aureus* by inhibiting growth and hemolysin and exotoxin production (8) and by inducing protein A expression (9), which prevents recognition of *S. aureus* by macrophages and neutrophils (10). *S. aureus* detected by the immune system trigger macrophages signalling pathways (11) [1].

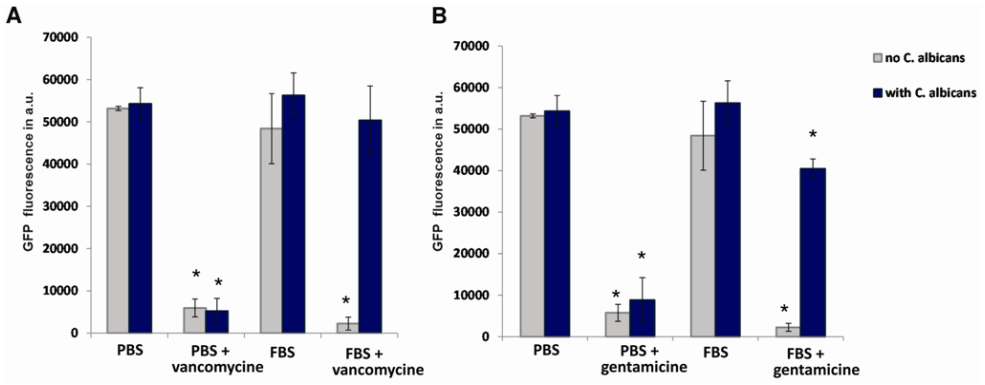

Figure 2 Effect of serum (FBS) and presence of *C. albicans* SC5314 hyphae on *S. aureus* NCTC8325-4^{GFP} vancomycin (A) and gentamicin (B) tolerance. After co-adhesion of *C. albicans* hyphae and *S. aureus* NCTC8325-4^{GFP} with or without FBS at 37°C for 3.5 h in PBS, pH 7, the plates were washed, and growth media (mixture of 30% tryptone soy broth and 70% nitrogen base acids pH 7, containing 0.5% D-glucose) and vancomycin or gentamicin added. The plates were incubated for an additional 18 h. The plates were washed to remove non-adherent cells, and growth was determined using a Fluorstar OPTIMA spectrophotometer. Experiments were performed in triplicate, and presented as average ± standard deviations over three independent experiments with separately cultured strains. Values that were significantly different ($P < 0.05$) in absence or presence of antibiotics are indicated (*).

In the oro-pharyngeal cavity however, more interesting phenomena can be observed with respect to interactions between Candida and different bacterial strains. In laryngectomized patients for instance [5], Candida species and oral and skin bacterial strains jointly colonize silicone rubber voice prostheses [6]. This colonization can lead to mal-functioning of the valve mechanism and ingrowth of hyphae into the silicone rubber, affecting its visco-elastic properties. In several patients, most notably those with *Rothia dentocariosa* as a bacterial species in combination with *C. albicans*, failure occurred within four months [7], while it has been suggested that the presence of lactobacilli would yield elongated life-times of these prostheses [5]. Recently, Buijssen [5] observed (see Table 1) that the presence of *R. dentocariosa* reduced the presence of Candida, while the presence of lactobacilli enhanced Candida presence. Since high Candida prevalence in the

presence of lactobacilli is at odds with expectations based on the life-time of prostheses, we decided to look whether *R. dentocariosa* stimulated yeast to hyphae transition more than lactobacilli (see Figure 3).

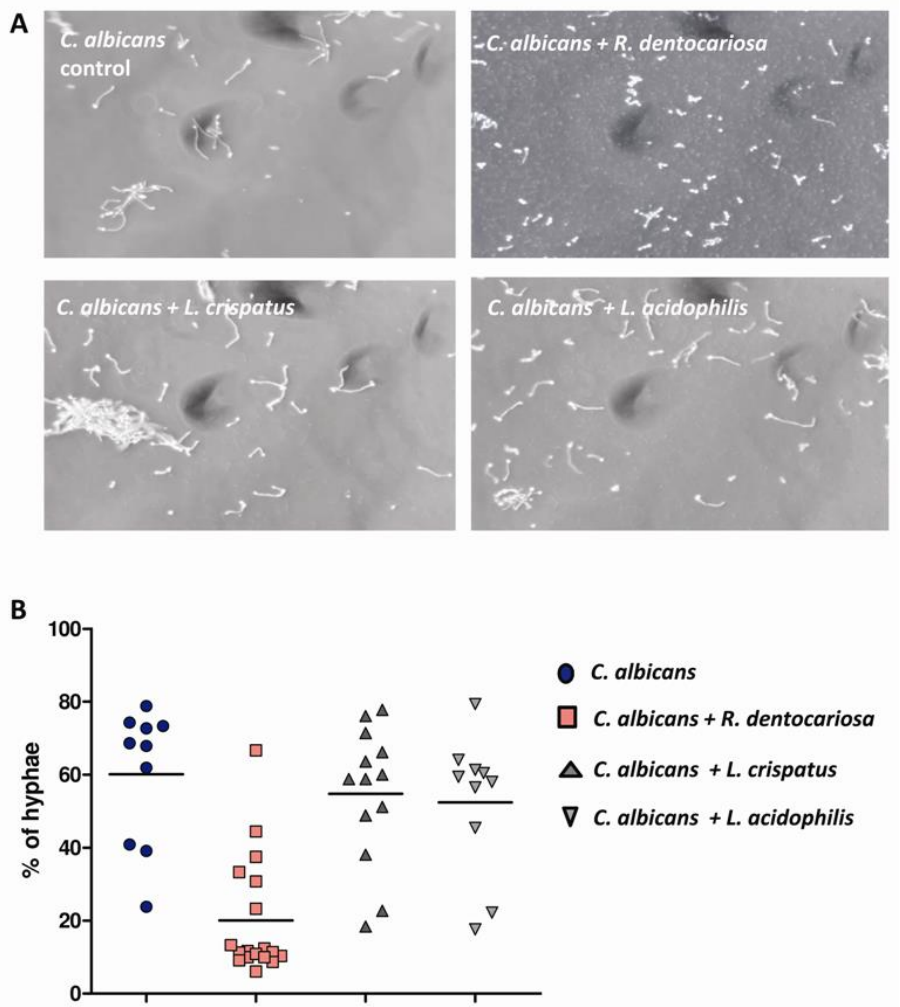

Figure 3 Microscopic analysis of inter-species interaction.
(A) Examples of phase-contrast microscopic images of the interaction between *C. albicans* and different bacterial strains.
(B) The percentage of *C. albicans* hyphae developed after co-culturing in presence of different oro-pharyngeal bacterial strains. Each dot corresponds to a value that was calculated based on one image. Images were taken over three experiments with separately cultured organisms.

Table 1. Number of CFU/cm^2 (bacteria and yeast) grown on silicone rubber in an eight days' time period after inoculation with a combination of a bacterial strain and a *C. albicans* or *C. tropicalis* strain, together with the percentage prevalence of the yeast in the final biofilm (Table taken from Buijssen [5]).

Yeast strain combined with	CFU/cm^2	*C. albicans* (%)	*C. tropicalis* (%)
Rothia dentocariosa	$4.4x10^5 \pm 0.4 x 10^5$	1.1	
Streptococcus salivarius	$3.5x10^5 \pm 0.3 x 10^5$	15.7	
Staphylococcus epidermidis	$1.7x10^6 \pm 1.3 x 10^6$	3.2	
Staphylococcus aureus	$2.4x10^4 \pm 1.2 X 10^4$	3.4	
Lactobacillus casei	$5.0x10^5 \pm 0.5 x 10^5$	45.4	
Lactobacillus fermentum	$7.2x10^5 \pm 1.5 x 10^5$	18.4	
Lactobacillus acidophilus	$1.3x10^5 \pm 0.3 x 10^5$	74.7	
Lactobacillus crispatus	$9.2x10^4 \pm 1.0 x 10^4$	36.5	
Lactobacillus johnsonii	$3.5x10^5 \pm 0.5 x 10^5$	84.4	
Rothia dentocariosa	$5.0x10^6 \pm 0.5 x 10^6$		0.3
Streptococcus salivarius	$9.0x10^5 \pm 0.6 x 10^5$		0.6
Staphylococcus epidermidis	$4.7x10^6 \pm 0.3 x 10^6$		5.4
Staphylococcus aureus	$2.8x10^7 \pm 0.7 x 10^7$		0.3
Lactobacillus casei	$2.0x10^7 \pm 0.3 x 10^7$		27.2
Lactobacillus fermentum	$4.9x10^6 \pm 1.0 x 10^6$		9.5
Lactobacillus acidophilus	$6.2x10^6 \pm 0.1 x 10^6$		8.9
Lactobacillus crispatus	$1.5x10^6 \pm 0.3 x 10^6$		34.9
Lactobacillus johnsonii	$4.4x10^5 \pm 0.4 x 10^5$		65.5

Hyphae grow into the silicone rubber [8]. Interestingly, the opposite result was found, illustrating the complexity of bacterial-fungal interactions.

Note that Noverr et al. [9] reported that lactobacilli decreased hyphae formation, but these results were obtained under different culturing conditions, such as growth medium, temperature and incubation time. Considering the impact that mixed species biofilms on voice prostheses have on the life-time of voice prostheses and the quality of life of laryngectomized patients, it would be worthwhile to further investigate cross-kingdom interactions between bacterial and fungal species in voice prosthetic biofilms.

Summarizing, a better understanding of the mechanisms underlying interkingdom microbial interactions will potentially lead to better therapeutic strategies and prevent or impede the development of polymicrobial diseases within an human host.

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Summary

In nature, microorganisms not only form mono-species biofilms, but they also co-exist within complex polymicrobial communities, containing both commensals and opportunistic pathogens. **Chapter 1** gives an overview of physical and chemical interactions occurring between fungi and bacteria within polymicrobial communities. For survival and reproductive success, interacting organisms in polymicrobial communities often take benefit of each other's presence. Additionally, microbial pathogens have developed sophisticated cell–cell communication systems termed quorum sensing (QS), that may or may not require direct physical contact between the organisms. The bacterial strains *Pseudomonas aeruginosa* and *Staphylococcus aureus* and the fungus *Candida albicans* often play a role in infections in the human body. Despite the fact that fungal-bacterial interactions have been investigated extensively, there is still limited information on the interplay between physico- and bio-chemical mechanisms that mediate their interaction. Thermodynamic modeling can be used to evaluate the nature of the adhesion forces between two microbial surfaces by calculating the interfacial free energy of the interacting organisms. Direct measurement of adhesion forces between microorganisms is possible using atomic force microscopy (AFM). Therefore the aim of this thesis was to reveal physico-chemical and biological mechanisms involved in interactions between the eukaryotic pathogen, *C. albicans* and prokaryotic bacterial pathogens.

In **Chapter 2** the nature of the adhesion forces between *P. aeruginosa* and different *C. albicans* morphologies, based on surface thermodynamics and AFM, was investigated. Adhesion of *P. aeruginosa* to hyphae was always accompanied by strong adhesion forces, but adhesion did not occur to yeast cells. Surface thermodynamics and Poisson analyses of adhesion forces indicated that the outermost mannoprotein-layer on hyphal surfaces created favorable acid-base

conditions for adhesion, allowing close approach of *P. aeruginosa*. Removal of these proteins caused unfavorable acid-base conditions, preventing adhesion of *P. aeruginosa*. Yet, favorable acid-base conditions alone are insufficient for mediating adhesion of *P. aeruginosa* to hyphae. *P. aeruginosa* PA14 *lasI*, unable to produce $3OC_{12}HSL$, showed favorable acid-base conditions, but was considerably less adherent to hyphae. However, growth in presence of $3OC_{12}HSL$ restored adhesion. Concluding, the mannoprotein-layer on the hyphal surface ensures favorable interaction conditions through attractive Lifshitz-Van der Waals and acid-base forces, allowing *P. aeruginosa* to closely approach the hyphal surface and interact stereo-chemically with the fungal cell wall for actual adhesion forces to take place.

C. albicans and *P. aeruginosa* are able to form pathogenic polymicrobial communities. *P. aeruginosa* colonizes and kills hyphae, but is unable to attach to yeast. However, the precise mechanism by which *P. aeruginosa* interacts with hyphae remains unclear. The role of *P. aeruginosa* chitin-binding protein (CbpD) in its physical interaction with *C. albicans* hyphae or yeast, based on surface thermodynamic and AFM analyses was evaluated in **Chapter 3**. A *P. aeruginosa* mutant lacking CbpD was unable to express strong adhesion forces with hyphae (-2.9 nN) as compared with the parent strain *P. aeruginosa* PAO1 (-4.8 nN) and showed less adhesion to hyphae. Also blocking of CbpD using N-acetyl-glucosamine yielded a lower adhesion force (-4.3 nN) with hyphae. Strong adhesion forces were restored after complementing the expression of CbpD in *P. aeruginosa* PAO1 *cbpD* yielding an adhesion force of -5.1 nN. Regardless of the absence or presence of CbpD on the bacterial cell surfaces, or their blocking, *P. aeruginosa* experienced favorable thermodynamic conditions for adhesion with hyphae, which were absent with yeast. In addition, adhesion forces with yeast

were less than -0.5 nN in all cases. Concluding, CbpD in *P. aeruginosa* is responsible for strong physical interactions with *C. albicans* hyphae. The development of this interaction requires time due to the fact that CbpDs have to invade the outermost mannoprotein layer on the hyphal cell surfaces. In order to do this, thermodynamic conditions at the outermost cell surfaces have to be favorable.

The interaction between *C. albicans* and the Gram-positive bacterium *S. aureus* is another example of a bacterial-fungal interaction. The mechanism behind the preferential adhesion of *S. aureus* to *C. albicans* hyphae during mixed biofilm growth was studied in **Chapter 4**. Several *C. albicans* mutant strains were screened to identify the specific receptor on *C. albicans* hyphae recognized by *S. aureus*. The findings from these experiments implicated the *C. albicans* adhesin agglutinin-like sequence 3 (Als3p) in playing a major role in the adherence process as demonstrated with an immune assay. This association was quantitatively established using AFM where the adhesion force between two species was significantly reduced for a *C. albicans* mutant strain lacking *als3*. This was further confirmed by confocal laser scanning microscopy. In order to elucidate the implications of these *in vitro* findings in a clinically relevant setting, an *ex vivo* murine model of co-infection was designed using murine tongue explants. Fluorescent microscopic images revealed extensive hyphal penetration of the epithelium typical of *C. albicans* mucosal infection. Interestingly, *S. aureus* was only seen within the epithelial tissue when associated with the invasive hyphae. This differed from tongues infected with *S. aureus* alone or in conjunction with the *als3* mutant strain of *C. albicans*, where bacterial presence was limited to the outer layers of the oral tissue. Collectively, the findings generated from this study

identified a key role for *C. albicans* Als3p in mediating this clinically-relevant bacterial-fungal interaction.

Although bacterial adhesion to the different morphologies of *C. albicans* has been amply studied, possible differences in staphylococcal adhesion forces along the length of *C. albicans* hyphae have never been determined. Therefore in **Chapter 5** we hypothesized that the forces mediating *S. aureus* NCTC8325-4^{GFP} adhesion to hyphae vary along the length of *C. albicans* SC5314 and MB1 hyphae, as compared with adhesion to yeast cells. *C. albicans* hyphae were virtually divided into a "tip" (the growing end of the hyphae), a "middle" and a so-called "head" region (the yeast cell from which germination started). Adhesion forces between *S. aureus* NCTC8325-4^{GFP} and the different regions of *C. albicans* SC5314 hyphae were measured using AFM. Strong adhesion forces were found at the tip and middle regions of *C. albicans* hyphae, while much smaller adhesion forces were measured at the "head" region. Adhesion forces exerted by the "head" region were comparable with the forces arising from budding yeast cells. A similar regional dependence of the staphylococcal adhesion forces was found for the clinical isolate involved in this study, *C. albicans* MB1. These novel findings provide new insights in the intricate interkingdom interaction between *C. albicans* and *S. aureus*.

S. aureus and *C. albicans* are frequently isolated from mixed bloodstream infections and intravascular catheter-related infections. In order to mimic the clinical situation the influence of fetal-bovine-serum or individual serum proteins on staphylococcal adhesion towards abiotic (tissue-culture-polystyrene, TCPS) or partly biotic (TCPS with adhering hyphae of *C. albicans*) surfaces was investigated in **Chapter 6**. AFM was used to measure adhesion forces between *S. aureus* and

the abiotic and biotic surfaces. Adsorption of individual serum proteins like albumin and apo-transferrin to abiotic TCPS surfaces during 60 min, impeded development of strong adhesion forces as compared to fibronectin, while 60 min adsorption of proteins from fetal-bovine-serum yielded a decrease in adhesion force from -5.7 nN in phosphate-buffered-saline to -0.6 nN. Adsorption of albumin and apo-transferrin also decreased staphylococcal adhesion forces to hyphae as compared with fibronectin. During 60 min exposure to fetal-bovine-serum however, initial (5 min protein adsorption) staphylococcal adhesion forces were low (-1.6 nN), but strong adhesion forces of around -5.5 nN were restored within 60 min. This suggests for the first time that in whole fetal-bovine-serum exchange of non-adhesive proteins by fibronectin occurs on biotic *C. albicans* hyphal surfaces. No evidence was found for such protein exchange on abiotic TCPS surfaces. Staphylococcal adhesion of abiotic and biotic surfaces varied in line with the adhesion forces and was low on TCPS in presence of fetal-bovine-serum. On partly biotic TCPS, staphylococci aggregated in presence of fetal-bovine-serum around adhering *C. albicans* hyphae.

Finally, in **Chapter 7** we discuss the current models, methods and aspects of bacterial-fungal interaction, implications of the discoveries made in this thesis and provide a suggestion for future study on these interactions with respect to voice prosthetic biofilms.

Samenvatting

Micro-organismen vormen niet alleen enkel-soortige biofilms, maar ze komen ook voor als complexe gemeenschappen waarin meerdere soorten samenleven, zowel onschadelijke als opportunistische pathogenen. **Hoofdstuk 1** geeft een overzicht van de fysische en chemische interacties tussen gisten en bacteriën binnen deze polymicrobe gemeenschappen. Organismen in een polymicrobe gemeenschap maken gebruik van elkaars aanwezigheid om te overleven en zich voort te planten. Daarnaast hebben microbiële pathogenen een geavanceerd communicatie systeem ontwikkeld genaamd quorum sensing (QS), waarmee cel-cel communicatie mogelijk is, zelfs bij afwezigheid van fysiek contact tussen de organismen. De bacteriële stammen *Pseudomonas aeruginosa* en *Staphylococcus aureus* en het gist *Candida albicans* spelen vaak een belangrijke rol bij infecties in het menselijk lichaam. Ondanks het feit dat de interacties tussen gisten en bacteriën grondig zijn bestudeerd is er slechts gebrekkige informatie over hoe fysische en biochemische mechanismen samenwerken om deze interacties mogelijk maken. Met het gebruik van modellen uit de thermodynamica is het mogelijk de hechtingskrachten tussen microbiële oppervlakken te evalueren, door gebruik te maken van berekeningen omtrent de oppervlakte vrije energie van de betreffende organismen. De werkelijke hechtingskrachten tussen micro-organismen kunnen gemeten worden met behulp van atomaire kracht microscopie (AFM). Het doel van dit proefschrift is om erachter te komen welke fysisch-chemische en biologische mechanismen betrokken zijn bij de interactie tussen het eukaryote pathogeen *C. albicans* en prokaryote pathogenen.

In **Hoofdstuk 2** is de oorsprong van de hechtingskrachten tussen *P. aeruginosa* en de verschillende vormen van *C. albicans* onderzocht met behulp van oppervlakte thermodynamica en AFM. Hechting van *P. aeruginosa* aan hyfen ging in alle gevallen samen met sterke hechtingskrachten, terwijl geen hechting plaatsvond aan gistcellen. Oppervlakte thermodynamica en Poisson analyse van de

hechtingskrachten toonden aan dat de buitenste manno-eiwit laag op hyfe oppervlakken de voorwaarden creëerde voor gunstige zuur-base interacties, waardoor *P. aeruginosa* in staat was te hechten. Verwijdering van deze eiwitten gaven ongunstige zuur-base interacties waardoor de hechting van *P. aeruginosa* ook werd verhinderd. Alleen gunstige zuur-base interacties zijn niet voldoende om de hechting van *P. aeruginosa* aan hyfen te laten plaatsvinden. *P. aeruginosa* PA14 *lasI*, welke niet in staat is om $3OC_{12}HSL$ te produceren, liet gunstige voorwaarden voor zuur-base interacties zien, maar hechtte minder goed aan hyfen, terwijl groei in de aanwezigheid van $3OC_{12}HSL$ herstel van de hechting aan hyfen toonde. Kort gezegd zorgt de manno-eiwit laag op het oppervlak van de hyfe voor gunstige interactie condities door attractieve Lifshitz-Van der Waals krachten en zuur-base interacties, waardoor *P. aeruginosa* het hyfe oppervlak tot dichtbij kan naderen en stereo-chemisch met de gistcelwand contact kan maken waardoor sterke hechtingskrachten optreden.

C. albicans en *P. aeruginosa* zijn in staat tot het vormen van pathogene microbiële gemeenschappen. *P. aeruginosa* hecht aan en doodt hyfen, maar is niet in staat om aan gistcellen te hechten. Het precieze mechanisme waarmee *P. aeruginosa* zijn interacties met hyfen aangaat is echter niet bekend. De invloed van het *P. aeruginosa* chitin-binding protein (CbpD) op de interactie met *C. albicans* hyfe of gist is onderzocht op basis van oppervlakte thermodynamica en AFM in **Hoofdstuk 3**. Een *P. aeruginosa* mutant zonder CbpD toonde minder sterke hechtingskrachten met hyfen (-2.9 nN) dan de moederstam *P. aeruginosa* PAO1 (-4.8 nN) en liet minder hechting aan hyfen zien. Ook het blokkeren van CbpD met N-acetyl-glucosamine leverde een verlaging van de hechtingskrachten (-4.3 nN) met hyfen op. De hechtingskrachten herstelden na het complementeren van de expressie van CbpD in *P. aeruginosa* PAO1 *cbpD*, naar waarden van -5.1 nN. Onafhankelijk van de aanwezigheid van CbpD op het oppervlak van bacteriële

cellen, of het blokkeren ervan, bleven de thermodynamische condities voor hechting tussen *P. aeruginosa* en hyfen gunstig. Deze gunstige condities waren niet aanwezig tussen *P. aeruginosa* en gist, waarbij de hechtingskrachten in alle gevallen lager waren dan -0.5 nN. Hieruit concluderen we dat CpbD in *P. aeruginosa* verantwoordelijk is voor de sterke fysische interactie met *C. albicans* hyfe. Het tot stand komen van deze interactie kost tijd doordat CpbDs eerst de buitenste manno-eiwit laag op het hyfe oppervlak moeten binnendringen. Om dit mogelijk te maken moeten de thermodynamische condities op het buitenste cel oppervlak gunstig zijn.

De interactie tussen *C. albicans* en de Gram-positieve bacterie *S. aureus* is een ander voorbeeld van bacterie-gist interacties. Het mechanisme achter de voorkeur van *S. aureus* om in gemengde biofilms aan *C. albicans* hyfe te hechten is bestudeerd in **Hoofdstuk 4**. Verschillende *C. albicans* stammen werden bekeken om de specifieke receptor op *C. albicans* hyfe te ontdekken die herkend wordt door *S. aureus*. De bevindingen van de experimenten duiden erop dat *C. albicans* een adhesine genaamd “agglutinin-like sequence 3” (Als3p) bevat dat een grote rol speelt in het hechtingsproces, wat werd aangetoond met een immuun test. Deze associatie is kwantitatief vastgesteld met behulp van AFM waarbij de hechtingskrachten tussen *S. aureus* and *C. albicans* significant lager was voor een *C. albicans* mutant zonder *als*3. Dit werd verder bevestigd door confocale laser scanning microscopie. Om deze *in vitro* resultaten in een klinisch relevante setting te bevestigen, is een *ex vivo* co-infectie muismodel opgesteld, waarbij muizentongen werden gebruikt. Fluorescentie microscopie toonde aan dat hyfen in staat waren het epithelium binnen te dringen, iets wat kenmerkend is voor *C. albicans* slijmvlies infecties. Opvallend genoeg was *S. aureus* alleen aanwezig in het epitheel weefsel wanneer deze geassocieerd was met de invasieve van hyfen. In tegenstelling tot muizentongen die alleen met *S. aureus* of samen met de *als*3

mutant van *C. albicans* geïnfecteerd waren, waren bacteriën enkel aanwezig op de buitenste lagen van het orale weefsel. Samenvattend, de bevindingen van deze studie duiden op een hoofdrol voor *C. albicans* Als3p in het mediëren van deze klinisch relevante interactie tussen bacterie en gist.

Hoewel bacteriële hechting aan de verschillende vormen van *C. albicans* ruimschoots is bestudeerd, zijn mogelijke verschillen in hechtingskrachten van stafylokokken over de lengte van *C. albicans* hyfe nooit bepaald. Daarom is in **Hoofdstuk 5** de hypothese opgesteld dat de krachten die verantwoordelijk zijn voor de hechting van *S. aureus* NCTC8325-4^{GFP} aan hyfen, variëren over de lengte van *C. albicans* SC5314 en MB1 hyfe, vergeleken met de hechting aan gistcellen. *C. albicans* hyfen werden opgedeeld in een "top" (het groeiende eind van de hyfe), een "midden" en een "kop" sectie (de gistcel waaruit de hyfe ontkiemt). Hechtingskrachten tussen *S. aureus* NCTC8325-4^{GFP} en de verschillende secties van *C. albicans* SC5314 hyfen werden gemeten met AFM. Er werden sterke hechtingskrachten gemeten tussen *S. aureus* en de "top" en "midden" secties van *C. albicans* hyfen, terwijl de gemeten hechtingskrachten aan de "kop" veel kleiner waren. Hechtingskrachten tussen *S. aureus* NCTC8325-4^{GFP} en de "kop" waren vergelijkbaar met de hechtingskrachten gemeten op kiemende gistcellen. Vergelijkbare resultaten waarbij de hechtingskrachten van stafylokokken afhingen van waar op de hyfe interactie plaatsvond, zijn gevonden met de klinnische stam, *C. albicans* MB1. Deze verassende bevinden geven nieuw inzicht in de complexe interactie tussen *C. albicans* en *S. aureus*.

S. aureus en *C. albicans* worden regelmatig geïsoleerd uit gemixte bloedbaan infecties en intravasculaire katheter gerelateerde infecties. Om de klinische situatie na te bootsen is de invloed van foetaal kalfsserum en van individuele serum eiwitten op de hechting tussen stafylokokken en a-biotische (weefselcultuur polystyreen, TCPS) of gedeeltelijk biotische (TCPS met daarop

hyfen van *C. albicans*) oppervlakken onderzocht in **Hoofdstuk 6**. AFM werd gebruikt om de hechtingskrachten tussen *S. aureus* en de a-biotische en biotische oppervlakken te meten. Adsorptie van individuele serum eiwitten zoals albumine en apo-transferrine op a-biotisch TCPS gedurende 60 min verhinderde de ontwikkeling van sterke hechtingskrachten in vergelijking met fibronectine, terwijl 60 min lange adsorptie van eiwitten afkomstig van foetaal kalfsserum een daling in hechtingskrachten opleverde van -5.7 nN in PBS naar -0.6 nN. Adsorptie van albumine en apo-transferrine verlaagde ook de hechtingskrachten tussen stafylokokken en hyfen in vergelijking met fibronectine. Echter, tijdens 60 min durende blootstelling aan foetaal kalfsserum waren de initiële (5 min eiwit adsorptie) hechtingskrachten van stafylokokken laag (-1.6 nN), maar herstelden naar -5.5 nN binnen 60 min. Dit suggereert voor het eerst, dat in foetaal kalfsserum, ge-adsorbeerde eiwitten die hechting tegengaan worden vervangen door fibronectine op biotische *C. albicans* hyfe oppervlakken. Er werd geen bewijs gevonden dat hetzelfde proces plaatvond op a-biotisch TCPS. Hechting van stafylokokken aan a-biotische en biotische oppervlakken varieerde conform de hechtingskrachten en was laag op TCPS in de aanwezigheid van foetaal kalfsserum. Op gedeeltelijk biotisch TCPS vond, in de aanwezigheid van foetaal kalfsserum, aggregatie van stafylokokken rond *C. albicans* hyfen plaats.

Tenslotte bespreken we in **Hoofdstuk 7** de huidige modellen, methoden en aspecten van bacterie-gist interacties, implicaties van de ontdekkingen in dit proefschrift en dragen we suggesties aan voor toekomstige studies naar deze interacties met betrekking tot polymicrobe biofilms op stemprotheses.

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